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INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

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18TH INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY

PART II

Amsterdam, September 23–28, 1990

Guest Editors

U. A. Th. BRINKMAN
(Amsterdam)

H. POPPE
(Amsterdam)

The proceedings of the *18th International Symposium on Chromatography* are published in two consecutive volumes of the *Journal of Chromatography*: Vols. 552 and 553. The Preface to the proceedings only appears in Vol. 552; a combined Author Index to both Vols. 552 and 553 only appears in Vol. 553. The proceedings are dedicated to Professor **J. F. K. Huber**; on p. XV of Vol. 552 appears an introduction to his life and work.

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CHROMSYMP. 2200

High-performance liquid chromatography post-column derivatization with fluorescence detection to study the influence of ambroxol on dipalmitoylphosphatidylcholine levels in rabbit eustachian tube washings

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ABSTRACT

In this work an appropriate high-performance liquid chromatography method was set up to guarantee specificity, sensitivity, precision and accuracy in analyzing dipalmitoylphosphatidylcholine (DPPC) in rabbit eustachian tube washings, as well as to determine its varying levels after administration of ambroxol chloride. The procedure is based on a post-column derivatization with fluorescence detection using 1,6-diphenyl-1,3,5-hexatriene which exhibits increased fluorescence in a lipid environment. DPPC was chromatographed on a Hypersil C_{18}. The mobile phase for the isocratic elution consisted of 40 mmol/l choline chloride in methanol–tetrahydrofuran (97:3). Ambroxol was given to a group of New Zealand white rabbits at a dose of 30 mg/kg. A second group receiving vehicle only acted as controls.

INTRODUCTION

The existence of a surface-active agent in the mammalian eustachian tube has been proved and it has also been demonstrated that this surfactant functions as a surface tension-lowering substance in a manner similar to lung [1–6]. Literature data, in particular Hills [5] thin-layer chromatography (TLC) studies on dog and rabbit eustachian tube washings, show that tubal surfactant, as well as pulmonary surfactant [7–9], mainly consist of phosphatidylcholines (PCs); phosphatidylethanolamines (PEs) and sphingomyelins are present in lesser quantities [10].

In a previous work [11], dipalmitoylphosphatidylethanolamine was identified and determined in rabbit eustachian tube washings; in order to extend the research, PCs are now investigated.

As reported by Wheeler *et al.* [12], the eustachian tube synthetizes an elevated quantity of disaturated PCs and dipalmitoylphosphatidylcholine (DPPC) is either the major factor responsible for the surface-active properties [13] or is the predominating

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compound in tubal and lung washing [10]; the high percentage of palmitic acid in tubal washings (60% in comparison to 70% in lung washings) [14] confirms the presence of disaturated PCs in eustachian tube.

In secretory otitis media a decrease in tubal surfactant levels has been reported [15]; thus the administration of a drug which promotes surfactant secretion should be successful in treating this type of condition. Ambroxol chloride [16], 4-{[(2-amino-3,5-dibromophenyl)methyl]amino}cyclohexanol, is a drug with fluidizing activity on bronchial secretion; as it stimulates the secretion of alveolar surfactant, it is also supposed to stimulate the secretion of tubal surfactant. The aim of this work is to verify its effect on DPPC levels in both bronchial and eustachian secretions, by means of comparative studies on treated and control animals. This approach requires a specific, sensitive, accurate and precise analytical method for the determination of DPPC in biological matrix (tubal and lung washings).

EXPERIMENTAL

Materials

Standard DPPC was obtained from Sigma (St. Louis, MO, U.S.A.). 1,6-Diphenyl-1,3,5-hexatriene (DPH) and choline chloride were from Aldrich-Chemie (Steinheim, Germany). Methanol and tetrahydrofuran were from J. T. Baker (Deventer, The Netherlands). Physiologic saline was from Abbott (Aprilia, Italy) and ambroxol was from De Angeli (Milan, Italy). All reagents were high-performance liquid chromatography (HPLC) grade.

Equipment

Qualitative and quantitative determinations were performed on an HP 1090 M liquid chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with a sample valve (Model 7410 Rheodyne) with a 100-μl loop.

The post-column derivatization system consisted of a Milton-Roy minipump with pulse damper connected via a T-junction to a mixing chamber (30 cm $\times$ 0.5 mm I.D. TFE, filled with 250-μm glass beads), 10 ft. $\times$ 0.5 mm I.D. TFE tubing (Supelco, Bellefonte, PA, U.S.A.) maintained at 50°C with a water jacket and then a 1046 A fluorescence detector (Hewlett-Packard). Data were elaborated by a HP 9000 Model 310 work-station (Hewlett-Packard).

Analytical conditions

The column was an ODS Hypersil 100 mm $\times$ 4.6 mm I.D., 5-μm spherical particles (Hewlett-Packard). Isocratic elution was carried out with mobile phase A (40 μM choline chloride in methanol) and mobile phase B (tetrahydrofuran, 97:3, v/v) at a flow-rate of 1 ml/min.

The detection reagent was distilled water containing, per litre, 150 μl of 3 μM DPH solution in tetrahydrofuran and 0.001% (v/v) Tween 20, at a flow-rate of 1.2 ml/min. The detergent was necessary to prevent the build-up of background fluorescence due to adsorption of PC vesicles in the flow cell.

The excitation and the emission wavelengths were 340 and 460 nm, respectively.

Standard solution

The optimal analytical conditions were assessed in rabbit lung washings. Since the DPPC concentrations in lung and tubal washings differed considerably, it became necessary to prepare two calibration curves. Standard solution was prepared by dissolving DPPC in methanol and diluting this stock solution to obtain concentrations ranging from 500 to 3000 μg/ml (first curve) and from 5 to 300 μg/ml (second curve).

The detector response was linear within the ranges above described and the two regression equations were: $y = 0.0253x + 0.379$, $r = 0.991$ and $y = 0.228x + 7.76$, $r = 0.968$, respectively.

The precision of the chromatographic procedure was indicated by five replicate injections of standard solutions (250 and 1500 μg/ml DPPC). The standard deviations were ± 0.7 and ± 0.5, and the accuracies were 0.19 and 0.17%, respectively. The limit of detection was 1.5 μg/ml with a signal-to-noise ratio 2:1.

Biological samples

The biological samples were taken from New Zealand white rabbits (average weight: 3.5 kg) and the washing was carried out with physiologic saline as described in the literature [5,10,15].

Ambroxol (30 mg/kg) was given by means of intravenous administration to a group of twelve white New Zealand rabbits in order to verify the effect of the drug on DPPC levels. A second group of twelve rabbits acted as controls.

Sample preparation

A 10-ml volume of Eustachian tube washings (or lung washings) was centrifuged at 1000 *g* for 5 min in order to exclude the risk of washing contamination by erythrocytes and cell debris. The supernatant was transferred to a Petri dish and lyophilized. To the lyophilized sample were added 5 ml of chloroform. After filtration on Millipore HF filters (0.45 μm), the solution was dried under a stream of nitrogen. To the dry residue were added 500 μl of methanol. Triplicate 100-μl aliquots were directly chromatographed.

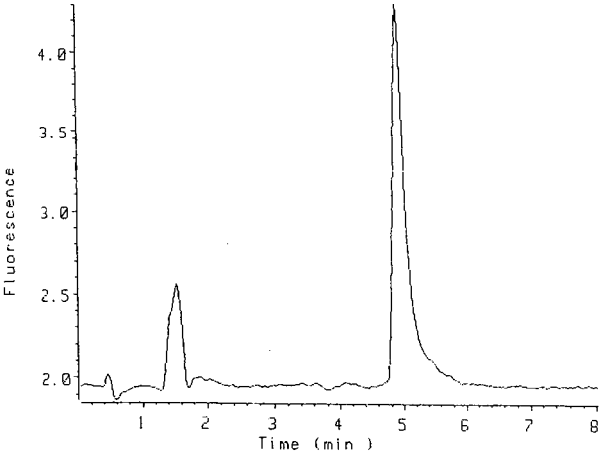

Fig. 1. Chromatogram of standard solution of DPPC (250 μg/ml).

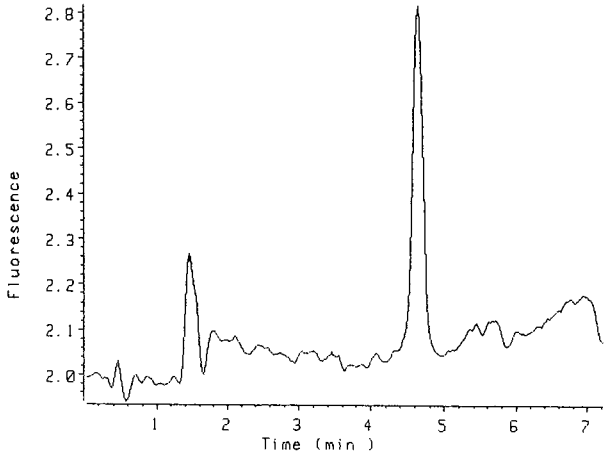

Fig. 2. Chromatogram of DPPC in eustachian tube washings.

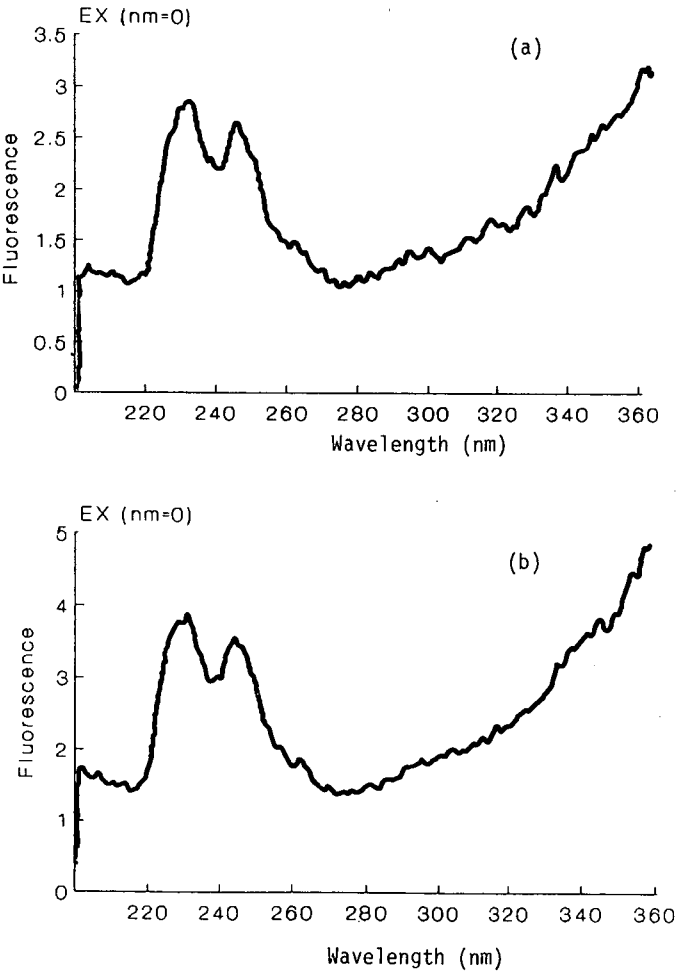

Fig. 3. Excitation spectra of DPPC standard solution (a) and biological sample (b).

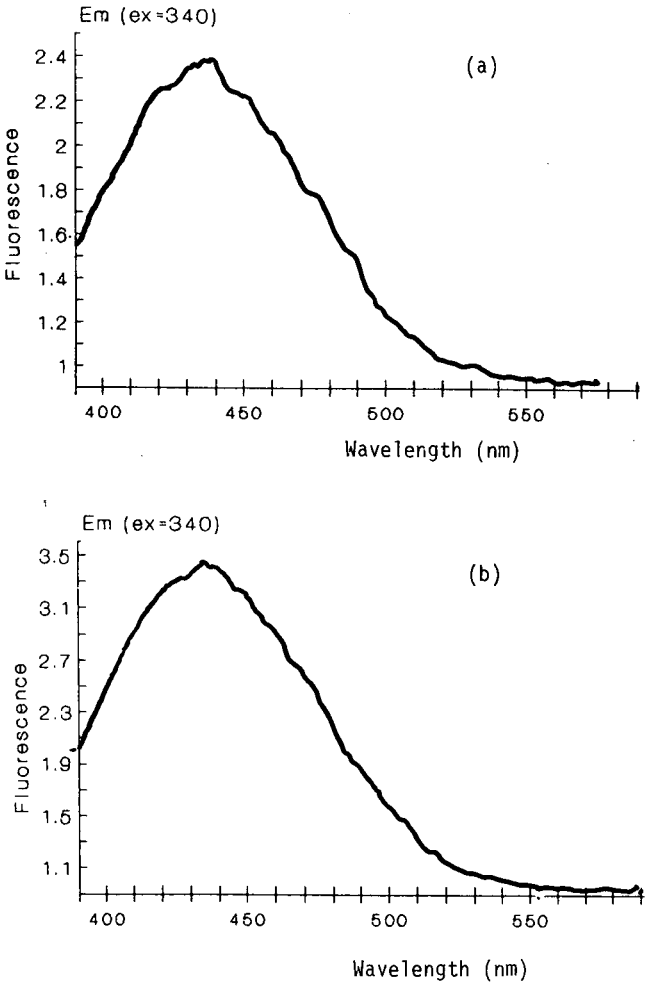

Fig. 4. Emission spectra of DPPC standard solution (a) and biological sample (b).

TABLE I

MEAN VALUES OF DPPC IN TWELVE RABBITS

Sample	Concentration (mean ± S.D.) (μg/ml)	t^a	P
Lung washings			
Control	787.78 ± 65.65	5.60	<0.001
Treated	1024.76 ± 131.11		
Eustachian tube washings			
Control	15.15 ± 1.07	10.63	<0.001
Treated	35.47 ± 6.53		

[a] t = Student's t-test.

RESULTS AND CONCLUSION

The starting point of the study was the method proposed by Postle [17] but it was found to be unsuitable for our purposes. Under the modified chromatographic conditions described above, DPPC gave a retention time of 5 min (Fig. 1); standard solutions of distearoyl- and palmitoyllinoleylphosphatidylcholine were chromatographed and the retention times (8.7 and 4.4 min, respectively) do not interfere with DPPC. Fig. 2 shows the chromatogram of DPPC in eustachian tube washings.

During the chromatographic analysis, excitation and emission spectra were recorded both for DPPC standard and biological sample (tubal washings). Spectra are shown in Figs. 3 and 4. The method was found to be rapid, sensitive, specific and accurate for the analysis of DPPC in the biological samples considered.

The mean concentrations (μg/ml) of DPPC in biological samples are reported in Table I together with statistical significance.

DPPC levels of treated rabbits were enhanced in comparison with the control values, both in lung washings and in eustachian tube washings. The extent of the enhancement was much higher in the second group.

These results confirm the phospholipid nature of tubal surfactant and suggest new therapeutic applications in the otorhinolaryngologic pathologies for drugs which stimulate the production of surfactant in the lung.

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CHROMSYMP. 2270

Short Communication

Analysis of sulthiame in serum by narrow-bore high-performance liquid chromatography

Comparison of direct sample injection with pre-column switching and extrelut extraction

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ABSTRACT

Two high-performance liquid chromatographic methods for the analysis of sulthiame in serum are described. In the first method direct injection of serum samples onto a 4 × 4 mm I.D. (C_{18}, 25 μm) precolumn in a column-switching device was used. After a purge step, the adsorbed analytes were eluted onto a 250 × 3 mm I.D. (C_{18}, 5 μm) narrow-bore column for chromatographic separation. In the second method the sample pretreatment was an Extrelut extraction with dichloromethane–propanol-2 (95:5). After evaporation of the solvents, the residue was dissolved in methanol. The chromatographic separation was carried out on the same analytical column as used in the column-switching method. Both sample pretreatment methods were compared with respect to their suitability of routine analysis of sera from patients also receiving other antiepileptic drugs.

INTRODUCTION

Sulthiame (tetrahydro-2-*p*-sulphamoyl-phenyl-2H-1,2-thiazine-1,1-dioxide) is a product of the condensation of a sulphonic acid and an amino group, with the elimination of water [1]. Sulthiame (SULT, trade name "Ospolot"] is used as an antiepileptic drug (AED) in the treatment of psychomotor and grand mal seizures [2]. Although it is seldom prescribed, we have received more than 200 serum samples containing this AED in the last year. It seems that Ospolot is again being prescribed in our epilepsy centre most often.

In addition to some gas chromatographic methods, only a few high-performance liquid chromatographic (HPLC) methods for the determination of SULT in body fluids were published in the late 1970s [2–5].

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The determination procedure of Berry *et al.* [2] seems to be the best developed. They found linearity in the concentration range 1.25–10 mg/l and were able to carry out a chromatographic separation of SULT and several other AEDs. Ethyltolylbarbituric acid (ETB) was used as an internal standard (I.S.). The same I.S. was used for our routine and research analyses of the common AEDs and their clinically relevant metabolites [6–8].

At first we developed a pre-column switching method for direct sample injection based on our system for the determination of drugs in serum and ultrafiltrates [9,10]. We mixed the serum samples with an appropriate buffer in order to lower protein binding and sample viscosity. The buffer included ETB as an I.S. in a concentration which was suitable for detection at a wavelength of 245 nm (UV maximum of SULT). We found linearity in the concentration range of SULT 1–40 μg/ml and a good reproducibility of the results.

But in some samples with additional AEDs we observed sample background peaks (supposedly endogenous compounds) which could interfere with the separation of SULT. We therefore developed an alternative method using serum extraction with Extrelut columns (diatomaceous earth) with which sample extracts free of the interference mentioned above were available in all cases.

MATERIALS AND METHODS

Apparatus

An HP 1090 LC with a workstation and column-switching software was obtained from Hewlett-Packard (Waldbronn, Germany). The precolumns used for the direct serum injection were LiChroCART guard columns (4 × 4 mm I.D.) filled with LiChrospher-100 RP-18 (25 μm) obtained from Merck (Darmstadt, Germany). The analytical column was a "narrow-bore" column (250 × 3 m I.D.) filled with Nucleosil-100 C_{18} (5 μm) supplied by MZ-Analysentechnik (Mainz, Germany).

Chemicals and reagents

Acetonitrile ChromAR was obtained from Promochem (Wesel, Germany), and all other reagents (including the Extrelut-1 columns) were obtained from Merck.

Sulthiame was obtained from Bayer (Leverkusen, Germany) and ETB from Aldrich (Steinheim, Germany).

Internal standard buffer. A 22-g aliquot of sodium dihydrogenphosphate monohydrate and 1 g of sodium azide were dissolved in 1000 ml of HPLC water to give an acidic buffer of pH 5. ETB (25 mg) was dissolved in 3 ml of methanol and mixed with the buffer.

Extraction solvent. Dichloromethane (950 ml) and propanol-2 (50 ml) were mixed.

Mobile phase buffer. A 1000-ml volume of 0.01% phosphoric acid was adjusted to pH 4.5 with a 10% triethylamine solution in water, mixed with acetonitrile (90:10 or 40:60, v/v), and filtered for storage at room temperature. With a minimum content of 10% acetonitrile the buffer mixtures are stable for a long time.

Calibration and control samples

SULT (10.0 mg) was dissolved in 5 ml of methanol and diluted with water

(containing 0.1% sodium azide to prevent the growth of micro-organisms at room temperature) to 100 ml in a measuring flask. From this stock solution (having a concentration of 100 μg/ml SULT) 40 ml, 20 ml, 10 ml, 5 ml and 1 ml were pipetted into 100-ml measuring flasks and filled up with the 0.1% sodium azide solution.

As control samples a 5% solution of bovine albumin in water with a concentration of 4 μg/m SULT and pooled patient samples were used. Portions of 500 μl were transferred to 2-ml vials and frozen at −18°C.

Sample preparation

A 500-μl portion (calibration samples, patient sera and thawed controls) was mixed with 1000 μl of the I.S. buffer.

Direct sample injection with pre-column switching

A volume of 100 μl of the above mixture was injected by the autosampler of the HPLC apparatus into the precolumn (see above), placed outside of the column heating chamber, and flushed with 0.01% phosphoric acid.

After washing for 2 min the valve was switched and the compounds retained were eluted onto the analytical column. The valve configuration was as given in ref. 14.

Extrelut extraction

A 1000-μl portion of the mixture (sample and I.S. buffer) was pipetted onto an Extrelut-1 column. After 10 min 5 ml of the extraction solvent (see above) were added. The solvents were evaporated under vacuum at 30°C and the analytes dissolved in 100 μl methanol. A 10-μl sample of the extract was injected into the same analytical column as used above.

Chromatographic parameters

The chromatographic separations were carried out as gradient elutions with a mobile phase consisting of acetonitrile and an alkylamine–phosphoric acid mixture [11].

The gradient–time profile is shown in Table I (A = direct sample injection with pre-column switching, B = Extrelut extracts). The stop time was set at 14.00 min (A) or 12.00 min (B) and the post time at 3.00 min. The flow-rate was 0.6 ml/min, the oven temperature 45°C and the detection wavelength 245 nm.

RESULTS AND DISCUSSION

First of all it was determined whether the common AEDs prescribed in our epilepsy centre and their metabolites (MBs) interfere with the chromatographic separation of SULT. As Table II shows, SULT is well separated from AEDs and their MBs, which could be found in the patient samples.

The next step was the investigation of the linearity of the calibration curve. Although SULT concentrations higher than 20 μg per millilitre of serum were not found in any of the patient samples, the calibration curve was measured with 40 μg/ml as the highest level of detection. The linearity was found to be sufficient in the range 1.0–40.0 μg/ml SULT with the slope of the regression line 0.997 and the coefficient of correlation of 0.998.

TABLE I

GRADIENT PROFILES OF THE 1090 LC FOR BOTH METHODS

A = Column-switching; B = Extrelut extracts; SDS = Solvent delivery system.

Time (min) SDS	Orthophosphoric acid (0.01%) I	Buffer–acetonitrile (%)	
		90:10 II	40:60 III
A			
0.00	100%	0	0
2.00	100%	0	0
2.01	0%	60	40
4.00	0%	60	40
9.00	0%	20	80
9.01	0%	60	40
B			
0.00	[a]	60	40
2.00	[a]	60	40
7.00	[a]	20	80
7.01	[a]	60	70

[a] Solvent delivery system A is shut off.

At the outset of our determinations of SULT in patient sera we used only the method of direct sample injection with pre-column switching. A lot of time for sample pretreatment can be saved using this method. Hence it can be recommended for pharmacological studies with Ospolot using volunteers who do not suffer from epilepsy, if it is checked that the blood of the volunteers contains no interfering compounds.

Direct sample injection is not recommended for the analysis of patient samples.

TABLE II

RETENTION TIMES RELATIVE TO ETB OF SULT, COMMON AEDs AND MBs

A = Taken from the column-switching method; B = taken from the Extrelut results.

Substance	Relative retention time	
	A	*B*
2-Ethyl-2-phenylmalone diamide (PEMA)	0.59	0.33
Ethosuximide (ET)	0.64	0.36
Primidone (PRI)	0.66	0.39
CBZ-10,11-diol (DIOL)	0.65	0.45
Sulthiame (SULT)	0.70	0.52
Phenobarbital (PB)	0.85	0.74
CBZ-10,11-epoxide (EPO)	0.87	0.79
N-Desmethyl methsuximide (DM)	0.89	0.78
5-Ethyl-5-(*p*-tolyl) barbituric acid (ETB)	1.00	1.00
Phenytoin (PT)	1.05	1.11
Carbamazepine (CBZ)	1.07	1.21

Interfering sample background was found in the first half of the chromatograms of some of the blood samples of patients in our epilepsy centre. Sometimes the baseline was not clean in the retention time window of SULT. Similar observations were made earlier, when we developed a direct injection method for the analysis of the common AEDs in serum [9].

The reason might be that the C_{18} pre-column material is used for solid phase extractions when a large number of serum samples are injected. The same effect was not observed when solid phase extractions with Bond-Elut cartridges were used. For the off-line extractions the cartridges were used only once, whereas the reversed-phase (RP) material in the precolumn was loaded with many serum injections in sequence.

Some of the proteins injected with the serum samples are irreversibly adsorbed on the RP materials of the precolumn [12]. The surface of the packing in the precolumn is "protein-coated" and hence has a higher adsorption capacity than the freshly packed RP material. Unknown endogenous compounds in the patient samples (of sera and to an even higher degree of saliva samples [13]) are adsorbed in the column-switching methods. They are not observed, however, when using C_{18} cartridges off-line. These compounds can interfere with the analytes of interest in the first part of the chromatograms. These interferences can affect all substances eluted before phenobarbital in the separation of the AEDs.

The patients in the epilepsy centre Bethel are normally not on monotherapy, but take two, three or more AEDs and additional co-drugs. For this reason direct sample injection with pre-column switching was, in spite of its time-saving property, not the best choice for our routine determinations of SULT in patient samples.

From our experience of the different off-line extraction procedures available, the sample extraction using Extrelut clean-up columns affords the cleanest drug extracts from biological samples. Thus we developed an extraction using Kieselguhr as an alternative to direct sample injection for the determination of SULT.

The chromatographic parameters for the separation of the analytes in the solvent extracts remained basically the same as for the column-switching method (see Materials and methods section). Nevertheless, the chromatograms look slightly different (compare Figs. 1 and 2), because in one case the compounds were flushed

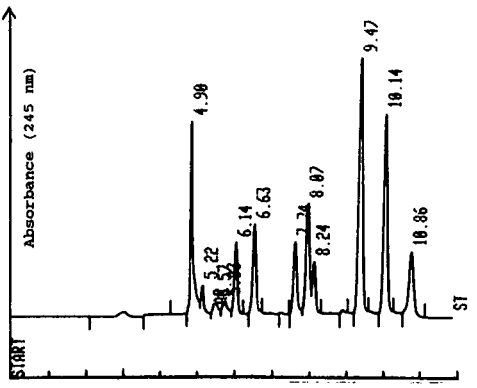

Fig. 1. Chromatogram from the direct injection of a serum containing Ospolot and other AEDs. Numbers at peaks indicate retention times in min; SULT (4.7 μg/ml), 6.63 min; ETB = I.S., 9.47 min. For the relative retention times of other AEDs, see Table IIA.

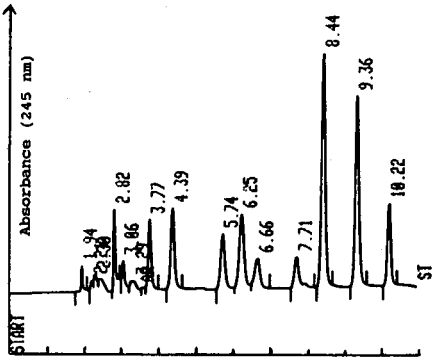

Fig. 2. Chromatogram from an Extrelut extract of the same serum as in Fig. 1. Numbers at peaks indicate retention times in min. SULT (4.8 μg/ml), 4.39 min; ETB = I.S., 8.44 min. For the relative RTs of other AEDs, see Table IIB.

through the precolumn in the straight-flush mode and in the other they were injected as methanolic solutions.

Recovery and reproducibility using Extrelut extraction

Since no control serum with SULT is commercially available, we made up control samples containing 4.0 μg/ml SULT with a bovine albumin matrix (5% in water). Portions of 500 μl were frozen at −18°C and thawed out before analysis of each of the sample series. In addition we checked the day-to-day precision of the analyses of a sample which was pooled from patient sera.

TABLE III

DAY-TO-DAY PRECISION OF A CONTROL AND A POOL SAMPLE ANALYSED USING THE EXTRELUT EXTRACTION METHOD

CS = Controle samples; PS = pool samples.

	n	Mean	S.D.	C.V. (%)
CS	58	3.95	0.164	4.15
PS	40	2.85	0.141	4.95

The method described is suitable for routine analyses having coefficients of variation which are lower than 5% for day-to-day determinations of SULT in both control and pooled samples. The mean value of 3.95 μg/ml which was found in the self-made control sample (see Table III) shows a 98.8% recovery of the spiked concentration of 4.0 μg/ml SULT.

CONCLUSION

Both of the methods described for the HPLC determination of sulthiame in

serum samples, direct injection with pre-column switching and solvent extraction using Extrelut columns, are in principle suitable for routine work.

In the case of samples from healthy volunteers taking SULT, the column-switching method gives the convenient option of automated sample pretreatment.

But, if the blood samples are taken from epileptic patients, especially from those on polytherapy with AEDs and other drugs, the Extrelut extraction, which yields cleaner extracts, is recommended for routine analyses.

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CHROMSYMP. 2274

Improved high-performance liquid chromatographic resolution of the geometric isomers of 6-hydroxy-4-(1-hydroxy-1-methylethyl)-1-cyclohexene-1-ethanol and by-products with β-cyclodextrin

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ABSTRACT

The aim of this study was to optimize the high-performance liquid chromatographic separation of (6*S*,4*R*)-(−)-6-hydroxy-4-(1-hydroxy-1-methylethyl)-1-cyclohexene-1-ethanol and its potential impurities to determine them in the active ingredient and in pharmaceutical formulations for purity and stability analysis. A comparison of conventional normal- and reversed-phase high-performance liquid chromatographic analysis and a method employing β-cyclodextrin was made. The reversed-phase analysis without β-cyclodextrin was undoubtedly unsuitable for an acceptable high-performance liquid chromatographic separation. On the other hand the other two methods were more selective and showed good precision, accuracy and linearity in the range investigated. However the use of β-cyclodextrin as eluent modifier with a reversed-phase was preferable for its improved selectivity and also for allowing the use of a non-toxic and less expensive eluent. Also, the limits of detection and quantitation obtained for the impurities made the β-cyclodextrin method very suitable for purity and stability analysis.

INTRODUCTION

(6*S*,4*R*)-(−)6-Hydroxy-4-(1-hydroxy-1-methylethyl)-1-cyclohexene-1-ethanol (CO/1408) is a polyol compound used in the treatment of some pulmonary diseases [1].

Diastereomeric and enantiomeric chromatographic separation of *cis* and *trans* and (+) and (−) *trans* forms of this compound has been achieved with β-cyclodextrin (β-CD), either adsorbed on to the stationary phase or dissolved in the mobile phase, as previously described [2]. Beside the *cis* isomer (II), two additional impurities might be present in the active ingredient: the starting manufacturing material Nopol (I), (1*R*)-(−)-6,6-dimethylbicyclo[3.1.1]hept-2-ene-2-ethanol, and the bicyclic degradation product (III), (6*R*, 7a*S*)-6-(1-hydroxy-1-methylethyl)-2,3,5,6,7,7a-hexahydrobenzofuran. Their structures, identified by nuclear magnetic resonance and mass spectrometric analysis, are shown in Fig. 1.

The present work describes the simultaneous determination of the active drug and its potential impurities by a rapid and inexpensive routine analysis employing β-CD as a selective eluent modifier.

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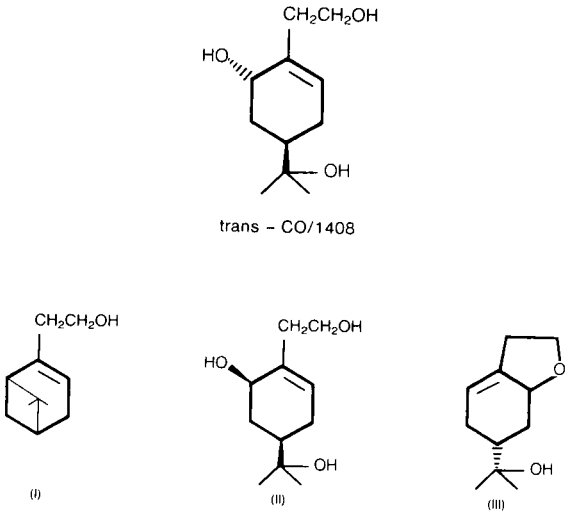

Fig. 1. Structures of *trans*-CO/1408 and its potential impurities: (I) Nopol, (II) *cis*-CO/1408 and (III) bicyclic degradation product.

A study of this inclusion complex separation in comparison with conventional normal- and reversed-phase high-performance liquid chromatographic (RP-HPLC) analysis is reported and discussed.

EXPERIMENTAL

A Varian 2010 liquid chromatograph with a 2050 variable-wavelength UV detector and a Perkin Elmer Series 4 liquid chromatograph with an LC-85 variable-wavelength UV detector were used. Spectrophotometric detections were recorded at 205 nm (range 0.32 a.u.f.s.). Experiments were carried out with a prepacked LiChrosorb reversed-phase RP-8 (10 μm) and a normal-phase Si_{60} (5 μm), 250 × 4.0 mm I.D. column (Merck, Darmstadt, Germany) at room temperature.

The eluents were prepared with Merck HPLC-grade solvents and were filtered and degassed prior to use. β-CD was of analytical reagent grade and supplied by Fluka (Buchs, Switzerland). CO/1408 and its impurities were synthesized in our laboratories, except Nopol, which was supplied by Fluka.

RESULTS AND DISCUSSION

A conventional normal-phase chromatographic system using an Si_{60} column and a non-aqueous eluent (*n*-hexane–diethyl ether–methanol 75:10:15) allowed a satisfactory baseline separation of the active ingredient CO/1408 and its potential impurities (see Fig. 2). However, the toxicity and expense of the mobile phase with a non-optimal separation of *cis* impurity induced us to develop an alternative RP chromatographic method.

Conventional RP analysis, using a LiChrosorb RP-8 (10 μm) column and an aqueous eluent was not suitable for an acceptable HPLC separation of the active

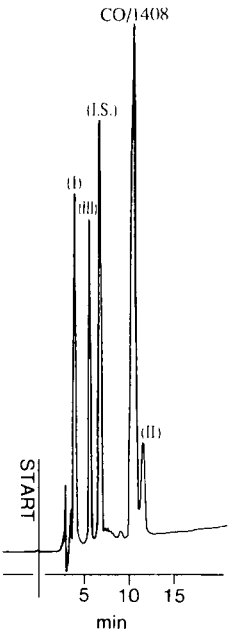

Fig. 2. Separation of CO/1408 and its potential impurities I, II and III using sobrerol (5-hydroxy-α,α,4-trimethyl-3-cyclohexene-1-methanol) as internal standard (I.S.) on a LiChrosorb Si_{60} (5 μm) 250 × 4.0 mm I.D. column. Mobile phase: *n*-hexane–diethyl ether–methanol (75:10:15). Flow-rate: 0.8 ml/min.

ingredient and its impurities, as shown in Fig. 3a. As a previous work [2] reported that β-CD is able to form a relatively stable complex with CO/1408, we decided to verify the utility of β-CD inclusion properties in this case also. The addition of β-CD to the mobile phase effectively decreased the retention times both of CO/1408 and of impurities, improving their separation in comparison with the above-mentioned conventional RP-HPLC analysis (see Fig. 3a and b).

As foreseen, an inversion of the elution order can be observed when comparing the normal-phase chromatogram with the reversed-phase chromatogram, with or without β-CD. Hence the more probable reaction impurity, that is the *cis* form of CO/1408, elutes first and is better quantified in RP than in normal-phase chromatography, where it is on the tail of CO/1408. This analytical method can therefore be used for quantitative CO/1408 determination without any interference with the three potential impurities and internal standard. In normal-phase analysis *trans*-sobrerol (5-hydroxy-α,α,4-trimethyl-3-cyclohexene-1-methanol) has been used as internal standard, while in RP analysis the inferior homologue of CO/1408 [6-hydroxy-4(1-hydroxy-1-methylethyl)-1-cyclohexene-1-methanol] has been employed. The two quantitative methods are both rapid and specific (see Figs. 2 and 3b).

Accuracy and precision were evaluated by ten injections of the same standard solution (3 μg of CO/1408 dissolved in the mobile phase) on two different days. Linearity was determined by six replications of five points between 2.5 and 3.7 μg and was significant for both methods. These results are summarized in Table I.

To evaluate the limit of detection and quantitation of the impurities, 80 μg of

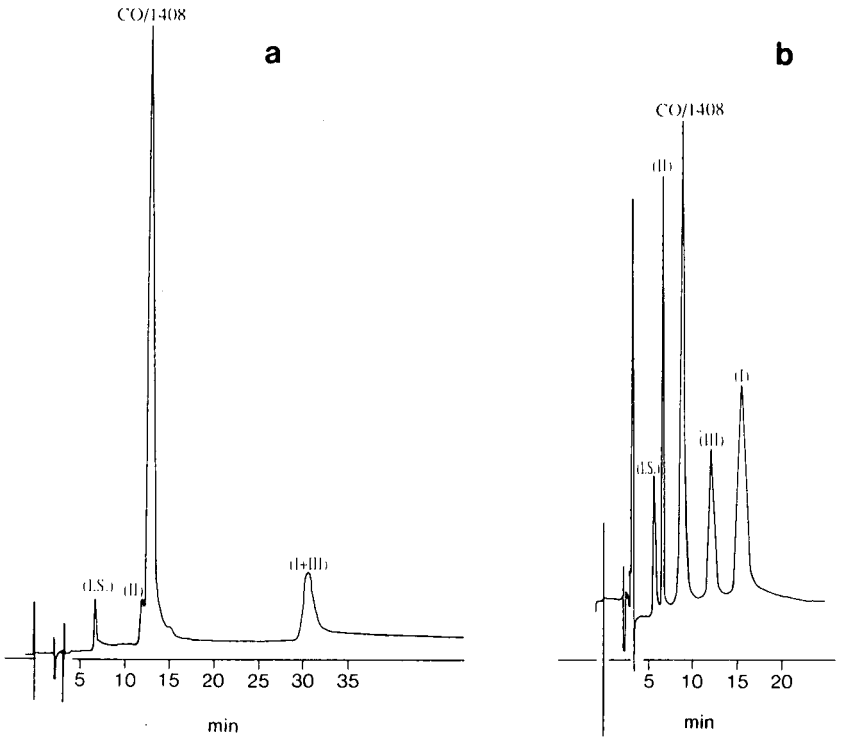

Fig. 3. (a) Separation of CO/1408 and its potential impurities I, II and III using CO/1470 [6-hydroxy-4-(1-hydroxy-1-methylethyl)-1-cyclohexene-1-methanol] as internal standard (I.S.) on a LiChrosorb RP-8 (10 μm) 250 × 4.0 mm. I.D. column. Mobile phase: potassium phosphate buffer solution (0.025 *M*, pH 7.4)–acetonitrile–methanol (90:5:5). Flow-rate: 1 ml/min. (b) As (a) but with the addition of β-CD to the mobile phase (1%, w/v).

CO/1408 were spiked with 0.1 μg of Nopol and bicyclic compound and 0.032 μg of *cis*-CO/1408. This mixture was dissolved in the eluent and injected with the β-CD HPLC method. The limits of detection and quantitation, evaluated for the three impurities, were therefore: Nopol and bicyclic compound < 0.15% (w/w) and *cis*-CO/1408 < 0.04% (w/w).

We can therefore conclude that the two methods are qualitatively and quantita-

TABLE I

Column phase	Precision[a] (%)	Accuracy[b] (%)	Intercept	r^c	F_{ratio} (ANOVA)
Normal	±1.93	+0.05	−0.020±0.181	0.997	2399.5 ($\alpha \ll 0.01$)
Reversed[d]	±1.65	+0.14	+0.059±0.088	0.999	11792.5 ($\alpha \ll 0.01$)

[a] Precision (%) = $\pm\ 100 \cdot t_{(0.05,v)}\ s / \bar{x}$ (v= degree of freedom).
[b] Accuracy (%) = $100\ (\bar{x} - \mu)/\mu$ (μ= true value).
[c] r = Coefficient of correlation.
[d] β-CD has been used as a selective eluent modifier.

tively comparable, but the analysis employing β-CD is preferable for a better HPLC separation of *cis*-CO/1408 and for the use of a non-toxic and less expensive eluent. Many authors have successfully used CDs for routine separations that are difficult to achieve by conventional methods [3–6]; this work is another significant example of the chromatographic application of β-CD in routine analysis.

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CHROMSYMP. 2324

Clean-up of plasma extracts by gel permeation chromatography during analysis of isosorbide nitrates by capillary gas chromatography

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ABSTRACT

This work describes how gel permeation chromatography (GPC) can be used for sample clean-up to reduce the fouling of the column in an automated on-column injector. The analytes were isolated from plasma together with the internal standard (isomannide dinitrate) by liquid–liquid extraction on Extrelut silica columns. The extracts were evaporated and reconstituted in tetrahydrofuran for separation of the analytes from non-volatile plasma components by GPC on a styrene–divinylbenzene column with 100 Å pore size. A programmable autosampler with an additional three-way valve was used for injection and fraction collection. The molecular weight fraction between 100 and 700 a.m.u. was collected and transferred to the on-column autosampler for capillary gas chromatography on a 30-m column butt-connected to a 0.2-m pre-column. The pre-column was replaced after 50 sample injections. When the GPC purification was excluded from the work-up procedure a deposit of non-volatile components was formed at the injection zone of the pre-column which resulted in excessive peak-tailing after only five or six injections of plasma extract. The limit of determination was 0.2 ng/ml plasma for isosorbide dinitrate and 0.4 ng/ml for the mononitrates.

INTRODUCTION

The vasodilator isosorbide dinitrate (ISDN) is rapidly metabolized in the liver and only a fraction of an oral dose reaches the systemic blood circulation as unchanged ISDN. Its primary metabolites isosorbide-5-mononitrate (ISMN-5) and isosorbide-2-mononitrate (ISMN-2) which are pharmacologically active are eliminated slower than the parent compound and circulate in the blood at higher concentrations [1]. The mononitrates are metabolized further to isosorbide and by glucuronidation to isosorbide-5-mononitrate-2-glucuronide [1]. Formation of small amounts of isomannide mononitrate and isoiodide mononitrate has also been reported [2]. For biopharmaceutical investigations it is necessary to quantify the analytes in plasma down to 1 ng/ml (ISDN) or a few ng/ml of the mononitrates.

ISDN has no intrinsic properties for sensitive detection by common detectors for liquid chromatography (LC). Detection limits of 2.5 ng for ISDN have been

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reported using post-column photolysis and electrochemical detection of the photolysis product nitrate [3]. This detection method has not been applied to biological samples and the sensitivity is not sufficient in the low ng/ml range. The thermal energy analyzer based on the chemiluminescence of nitrogen dioxide radicals produced by pyrolysis of the organic nitrates offers high sensitivity and an LC method for plasma samples has been described [4].

The most commonly used methods have been based on gas chromatography (GC) with electron-capture detection (ECD), first with packed columns [5,6] and later on capillary columns [2,7–9] without derivatization of the mononitrates. GC–tandem mass spectrometry (MS–MS) has also been used to determine isosorbide and isosorbide-5-mononitrate-2-glucuronide [10].

ISDN, chemically related to nitroclycerine and thermodynamically unstable, is sensitive to decomposition during GC and it is essential to have an inert and clean injector. When a non-selective extraction method such as liquid–liquid extraction is used, non-volatile sample components are co-extracted and deposited at the beginning of the column causing adsorption and resulting in non-linear calibration curves [7].

Sample purification by gel permeation chromatography (GPC) appears to be a logical method for elimination of non-volatile components with higher molecular weight than the analytes. One example is the GPC separation used on-line with GC for determination of polymer additives in polymers [11].

This work describes how GPC can be used in an automated manner for clean-up of plasma extracts prior to capillary GC of isosorbide nitrates.

EXPERIMENTAL

Chemicals and reagents

ISDN and ISMN-5 were obtained from Bofors Chemicals (Bofors, Sweden). ISMN-2 was obtained from Graesser Lab. (Sandycroof, Deeside, U.K.) and isomannide dinitrate was synthesized at the Department of Organic Chemistry at Kabi (Stockholm, Sweden). 2-Nitrobenzyl alcohol (NBA) was obtained from Fluka (Buchs, Switzerland). Dichloromethane and tetrahydrofuran, LiChrosolv grade, and disposable silica-gel columns, Extrelut-1, were obtained from Merck (Darmstadt, Germany). Dichloromethane was glass-distilled prior to use.

Instrumentation

Sample injection on the GPC column and fraction collection were performed in the same instrument by modification of a Gilson autosampler (Model 321, Gilson, Villiers-le-Bel, France). A three-way valve (Model LFYA; Lee Instac, Westbrook, CT, U.S.A.) was installed between the sampling needle and the syringe of the autosampler (dilutor/dispenser). The GPC column (300 mm × 7.8 I.D.) was packed with 10-μm Ultrastyragel with 100 Å pore size (Waters, Millipore, Milford, MA, U.S.A.). The eluent, tetrahydrofuran, was pumped at 1.5 ml/min with a Constametric pump (Model Bio 3000, LDC). The sample injections and fraction collections were all controlled by the programmable autosampler.

The gas chromatograph (Varian, Model 3500) was equipped with an on-column injector (Varian, Model 8035), an electron-capture detector and a GC-protector

(J & W Scientific, Folsom, CA, U.S.A.). A 0.2 m pre-column of the same type as the analytical column was butt-connected to a fused-silica column (30 m × 0.32 mm I.D.) coated with a 0.25-μm film thickness of the non-polar silicone phase DB-5 (J & W Scientific). Hydrogen was used as carrier gas at a flow-rate of 70 cm/s at 60°C and nitrogen was used as make-up gas (30 ml/min) to the detector, which was set to 250°C. The temperature programme of the injector and column is presented in Fig. 3. The peak areas were integrated by a Model 6000 laboratory data system from Nelson Analytical (Cupertino, CA, U.S.A.).

Sample preparation

Human blood samples were taken in heparinized Venoject tubes, cooled in an ice-bath and centrifuged within 30 min after sampling. The serum fraction was transferred to polypropylene tubes and the samples were stored at −20°C until analysis.

Plasma (0.9 ml) or blood diluted 1:2 with water was mixed with 0.1 ml internal standard solution (isomannide dinitrate, 50 ng/ml) and pipetted onto an Extrelut column. After 5 min, 6 ml dichloromethane were added to elute the analytes to a 10-ml glass-tube. The samples were put in an ice-bath and the solvent was evaporated with a stream of nitrogen. The residue in the tube was taken up in 0.2 ml terahydrofuran and transferred to micro-vials for the automatic injector. A 100-μl sample of the extract was injected onto the GPC column and the fraction between 6.9 and 8.4 ml was collected in another sample vial for the GC injector. The retention volume of the analytes was checked each day by injection of nitrobenzyl alcohol with UV detection at 254 nm. The retention volume of the analytes was 7.7 ml and the fraction between 6.9 and 8.4 ml was collected. The vial containing the collected fraction was manually transferred to the tray of the autosampler on the gas chromatograph, and 1 μl was injected for analysis.

Calibration was performed with spiked blank plasma purified along with unknown samples. The following levels were used for calibration; ISDN: 0.5, 1, 2.5, 3.5, 5.5, 9 and 10 ng/ml; ISMN-2: 2.5, 5, 10, 15, 25, 40 and 50 ng/ml; ISMN-5: 15, 25, 55, 80, 140, 225 and 275 ng/ml.

RESULTS AND DISCUSSION

Sample preparation

Liquid–liquid extraction on Extrelut columns was a convenient and rapid method for extraction of the analytes to dichloromethane from plasma. The solvent was evaporated on an ice-bath to avoid losses due to evaporation of the volatile analytes. The residue had to be taken up into tetrahydrofuran within 15 min after they had been blown to dryness to avoid losses due to evaporation of the analytes. The recovery of ISMN-2, ISMN-5 and ISDN in the extraction was checked in a separate experiment where the internal standard was added after the Extrelut extraction and evaporation steps, and the area ratios were compared with unextracted standards dissolved in tetrahydrofuran. Quantitative recovery was obtained for all of the analytes studied (see Table I). Calibration was performed by addition of the internal standard to spiked blank samples which resulted in linear calibration curves with good reproducibility between days (see Table II).

TABLE I

RECOVERY AND PRECISION

Compound	Concentration added (ng/ml)	Recovery (%)	n	Precision (R.S.D., %)	Detection limit (ng/ml)
ISDN	4.5	99.2	7	1.5	0.2
ISDN	50	101	8	2.8	
ISMN-2	4.5	98.9	7	2.7	0.4
ISMN-2	50	101	9	4.0	
ISMN-5	4.5	97.9	7	2.0	0.4
ISMN-5	50	103	9	2.5	

Stability of ISDN in blood and plasma

The stability of ISDN in fresh whole blood at 37°C was studied by adding ISDN and analyzing whole blood and plasma after different incubation times. The hematocrit of the blood was also measured to obtain the volume ratio of erythrocytes and plasma fractions. ISDN was rapidly distributed to blood cells within 1 min and then the concentration slowly decreased with approximately 25% reduction within 30 min (Fig. 1). The distribution coefficient between blood cells and plasma (C_c/C_p) was calculated to be 1.24 ± 0.02 (S.D.) from the blood concentrations, plasma concentrations and the hematocrit value according to the equation:

$$C_c/C_p = C_b\,(h \times C_p) - 1/h + 1$$

where C_c = ISDN concentration within the cell fraction, C_p = ISDN concentration in the plasma fraction, C_b = ISDN concentation in the whole blood and h is the cell fraction volume (hematocrit value %/100). The hematocrit value was 44% for the blood used in the experiment.

TABLE II

REPRODUCIBILITY OF CALIBRATION CURVES

Least-squares regression analysis of concentration (ng/ml) *versus* peak-area ratio of analyte and internal standard. A = Slope, B = intercept and r = correlation coefficient

Day	ISMN-2			ISMN-5			ISDN		
	A	B	r	A	B	r	A	B	r
1	0.713	0.008	0.9997	0.595	0.03	0.9997	0.901	−0.001	0.9999
2	0.704	0.02	0.9999	0.599	0.02	0.9996	0.905	−0.002	0.9999
3	0.715	0.005	0.9997	0.603	0.02	0.9999	0.923	0.000	0.9999
4	0.714	0.02	0.9999	0.592	0.02	0.9997	0.906	−0.002	0.9999
5	0.733	0.02	0.9991	0.614	0.01	0.9997	0.918	−0.002	0.9997
6	0.747	0.01	0.9998	0.613	0.03	1.0000	0.923	−0.002	0.9996
7	0.756	0.01	0.9975	0.612	0.01	0.9998	0.909	0.002	0.9993
8	0.689	0.01	0.9995	0.600	0.02	1.0000	0.907	0.000	1.0000
Mean	0.721	0.0129		0.604	0.02		0.912	−0.0008	
S.D.	0.0223	0.0061		0.0088	0.0076		0.0086	0.0014	

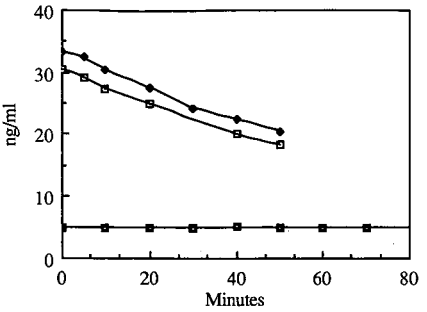

Fig. 1. Concentration of ISDN in blood (upper curve) and plasma (middle curve) after incubation at different times at 37°C, and the stability of ISDN in plasma at 22°C (lower line). Whole blood and plasma were spiked with ISDN to a concentration of 31 and 5.3 ng/ml, respectively.

A similar stability study was performed for ISDN in plasma at ambient temperature (22°C). ISDN was stable in plasma for at least 70 min (Fig. 1) ISDN is probably distributed to blood cells and hydrolyzed by esterases. Thus blood samples have to be cooled and centrifuged soon after sampling to obtain accurate results. Stability studies of frozen control samples showed that ISDN was stable at −20°C for at least 5 months.

GPC purification of plasma extracts

Initially we tried to inject the plasma extracts into the gas chromatograph after the Extrelut extraction and evaporation step but excessive tailing and adsorption problems occurred after only five or six injections. Tailing was especially pronounced

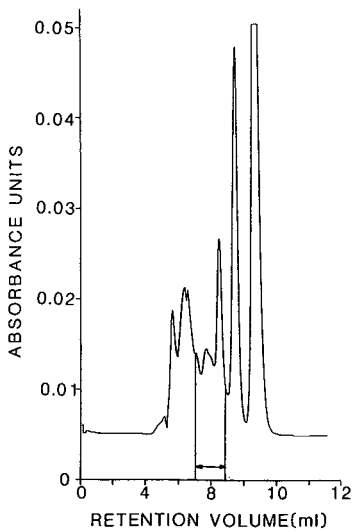

Fig. 2. UV chromatogram (254 nm) obtained after injection of 100 μl of plasma extract on the GPC column. The fraction between 6.9 and 8.4 ml was collected for analysis by GC.

for the mononitrates with a free hydroxy group. The problem with injector contamination has also been described by Ahnoff and Holm [7] and Lutz *et al.* [2] using splitless injection of plasma extracts. In general, cold on-column injection is supposed to be more gentle compared with splittless injection with flash evaporation of the sample, but the on-column injector was contaminated after fewer injections, probably due to a more concentrated zone of non-volatile material deposited at the beginning of the narrow column.

GPC purification of the extracts was then investigated for separation of the analytes from non-volatile plasma components such as lipids and other compounds with high molecular weight. The ultrastyragel column gave complete separation of toluene, ISDN and coenzyme Q_{10}. Coenzyme Q_{10} with a molecular weight of 862 was chosen as a representative of the lipoprotein fraction in plasma and was readily detected by UV for chromatographic registration. The internal standard isomannide dinitrate and the analytes co-eluted as one chromatographic peak, slightly broadened due to a minor difference in retention between the mono- and dinitrates. The elution fraction corresponding to a molecular weight of 100 to 700 a.m.u. (6.9–8.4 ml) gave quantitative collection of the analytes. A UV chromatogram of a plasma extract is presented in Fig. 2. Tetrahydrofuran was selected as the eluent for the GPC column to minimize hydrophobic interaction between sample components and the column packing material. This eluent was also a suitable solvent for the following step involving GC.

Capillary gas chromatography

Since it is known from previous work [7] that isosorbide dinitrates decompose at column temperatures above 150°C, hydrogen was chosen as the carrier gas to enable the use of higher flow-rates at a lower column temperature. The capillary column (DB-5) was of the same type as used by Lutz *et al.* [2], which is similar to the

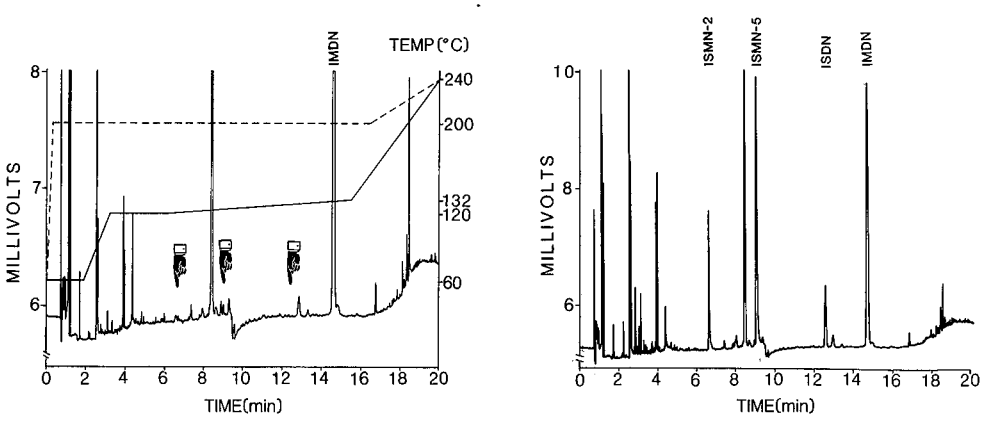

Fig. 3. GC profile obtained after injection of blank plasma sample. The injector temperature is shown by the broken line and the column temperature by the solid line. The hands show the retention time of the analytes in Fig. 4.

Fig. 4. GC profile obtained after injection of a patient plasma sample taken 2 h after a 10-mg oral dose of ISDN, with plasma of concentrations of 17.1, 70.6 and 8.5 ng/ml for ISMN-2, ISMN-5 and ISDN, respectively.

CPSil 5 column used by Ahnoff and Holm [7]. All the analytes were eluted within 16 min at a column temperature lower than 130°C at the conditions used in this work. The column gave good resolution of the analytes from plasma components (see Fig. 3 and 4) without any sign of analyte decomposition checked by variation of the residence time. Adsorption losses of the mononitrates were too low to be detected from the calibration curves which were linear in the concentration range of interest (see Table II).

The pre-column used as the injector liner was cut off from another column of the same type and replaced daily after 35–40 injections. The coated pre-column appeared to be more inert compared with an uncoated siloxane deactivated column because more samples could be injected without peak-tailing of the mononitrates.

Accuracy and presision

The recovery was close to 100% when determined at the 4.5 and 50 ng/ml level and the intra-assay precision was within 1–4% R.S.D. (see Table I). The inter-assay precision was 6.6% (5.2 ng/ml ISMN-2), 3.7% (28 ng/ml ISMN-5) and 4.7% (1.2 ng/ml ISDN). The method detection limit at the 99% confidence level was determined as three times the standard deviation of the concentration at a low level (Table I). The detection limit was 0.4 ng/ml for the mononitrates and 0.2 ng/ml for ISDN but can be decreased further by concentration of the GPC fractions.

CONCLUSIONS

Isolation of drugs and other small molecules from complex plasma extracts by GPC could be of general value as a purification method prior to capillary GC. The GPC purification in this application made it possible to inject at least 50 samples without replacement of the injector liner. ISDN was hydrolyzed in whole blood and samples had to be cooled and centrifuged within 30 min after sampling.

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CHROMSYMP. 2284

High-performance liquid chromatographic method for the determination of RGH-5702 in plasma samples

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ABSTRACT

A quick and selective high-performance liquid chromatographic method has been developed for the determination of RGH-5702 in plasma samples. A simple one-step extraction is used followed by reversed-phase chromatography and UV detection. This method allowed the separation of the compound and internal standard within 7 minutes. Validation of the method was performed prior to the assay of samples and continued throughout the study. Acceptable accuracy and precision was achieved at all concentrations investigated. The quantitation limit was 20 ng/ml using 1 ml of plasma. The method has been applied to the analysis of plasma samples from toxicokinetic studies in dogs.

INTRODUCTION

3-(2-Nitrophenylmethyl)-2-thiazolidinone (RGH-5702) is a new cytoprotective agent synthesized by the Chemical Works of Gedeon Richter. According to pharmacological investigations the compound has an inhibitory effect against indomethacin-induced gastric ulcer and decreases the acid secretion in pylorus ligated rats [1]. As part of the development of the compound it was necessary to develop a simple and selective method for the determination of the compound in biological fluids in order to perform a toxicokinetic study on dogs at three dose levels corresponding to the 28-day toxicity study.

EXPERIMENTAL

Chemicals

RGH-5702 (Fig. 1) and the internal standard (Fig. 1) were supplied by the Chemical Works of Gedeon Richter. Ethanol (HPLC grade) and diethyl ether (spectroscopy grade) were purchased from Merck (Germany). Glycocoll and sodium hydroxide (analytical purity) were obtained from Reanal (Hungary) and methanol (HPLC grade) from Aldrich (U.S.A.).

Chromatographic equipment and conditions

Chromatography was carried out with a Model 303 solvent delivery system, a

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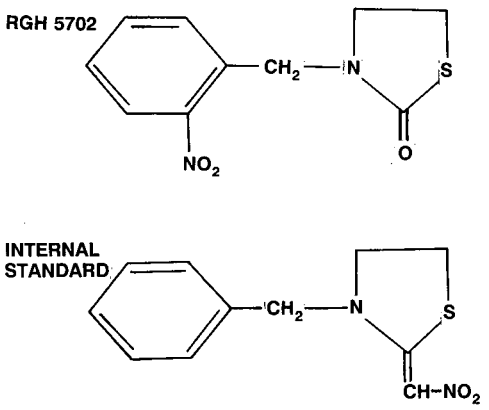

Fig. 1. Chemical structures of RGH-5702 [3-(2-nitrophenylmethyl)-2-thiazolidinone] and the internal standard (3-benzyl-2-nitro-3-methylene thiazolidine).

Model 116 variable-wavelength detector, a Model 231 sample injector, Dilutor 401, all from Gilson (France) and a COPAM PC-501 Turbo Computer (U.S.A.).

A LiChrosorb RP-8 column 200 × 4.6 mm I.D., particle size 5 μm (Hewlett-Packard) was used with methanol–doubly distilled water (60:40, v/v) as mobile phase at a flow-rate of 1.0 ml/min. Detection was carried out at 265 nm. High-performance liquid chromatographic (HPLC) analysis was performed at room temperature (*ca.* 25°C).

Preparation of plasma calibration samples

A stock solution of RGH-5702 was prepared in ethanol (10 mg/100 ml). A series of plasma calibration samples containing the compound in equal concentrations of 20, 40, 100, 200, 500 ng/ml were prepared from stock solution and pooled dog plasma. For example 10.0 ml of pooled dog plasma was spiked with 10 μl of stock solution to give a final concentration of 100 ng/ml for RGH-5702. These plasma calibration samples were divided into 1 ml aliquots stored at −18°C and used to construct the calibration curves.

General assay procedure

A stock solution of internal standard (3-benzyl-2-nitro-3-methylene-thiazolidine) was prepared in chloroform (10 mg/50 ml). A working solution of internal standard was prepared by diluting 1 ml of the stock solution with chloroform to a final concentration of 400 μg/ml.

A volume of 10 μl of the internal standard working solution was dried into a test tube into which 1 ml of dog plasma, 0.2 ml of gly–NaOH buffer (1.0 *M*, pH 10.0) were added and shaken with 7.0 ml of diethylether for 10 min. After refrigerated centrifugation (10 min at 1500 *g*, 4°C) the organic layer was removed to a new test tube and evaporated to dryness under a stream of nitrogen at approximately 40°C. The residue was redissolved in eluent (100 μl) by vortexing and sonication.

An aliquot (20 μl) was injected into the HPLC system.

RESULTS AND DISCUSSION

Chromatography

Chromatograms from the assay of dog plasma are given in Fig. 2. Significant interfering peaks of endogenous compounds were not recorded on the chromatogram. The described method allowed the separation of the compound and the internal standard within 7 min. The chromatographic system is very simple and requires

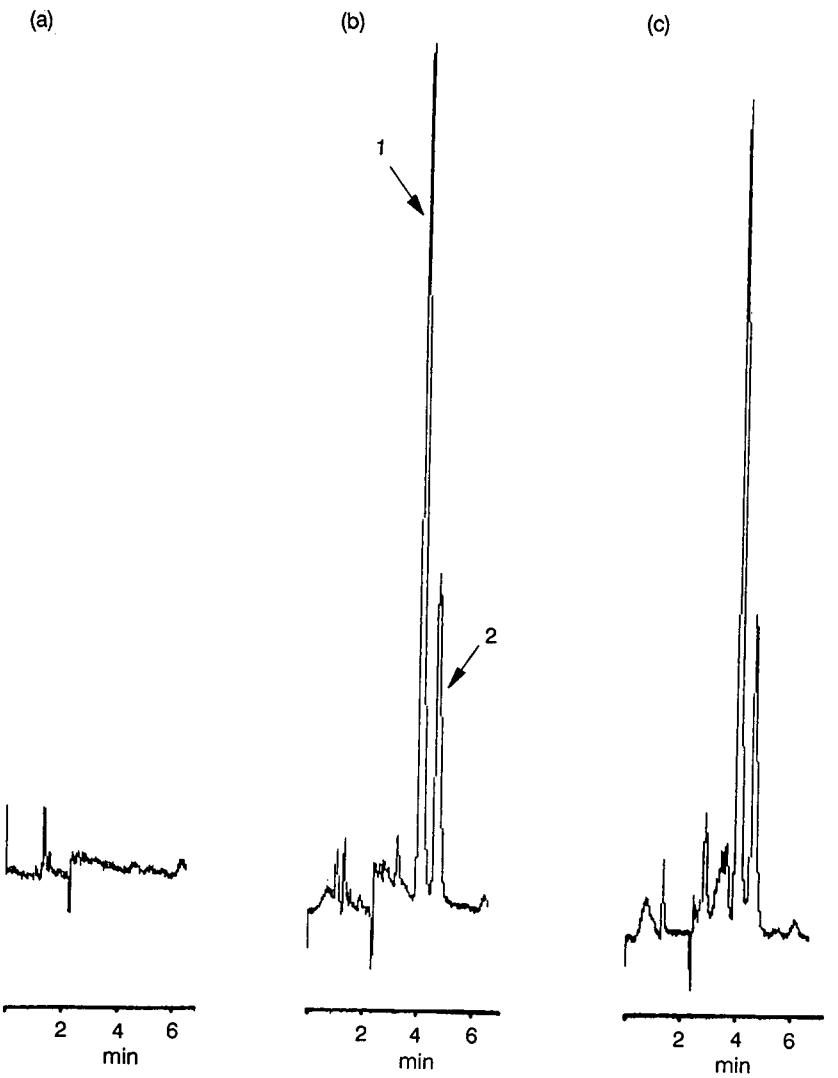

Fig. 2. Chromatograms of extracted dog plasma samples: (a) blank plasma, (b) extracted dog plasma calibration standard containing 200 ng/ml RGH-5702 (1 = internal standard, 2 = RGH-5702), (c) extracted plasma samples with internal standard 1 h after oral administration of 10 mg/kg of RGH-5702.

TABLE I

WITHIN-DAY AND BETWEEN DAYS PRECISION OF HPLC PROCEDURE FOR RGH-5702 IN DOG PLASMA

Concentration (ng/ml)	Peak area ratio (mean ± S.D. $n = 4$)	Coefficient of variation (%)
Within-day precision ($n = 4$)		
40	0.185 ± 0.005	3.1
200	0.520 ± 0.029	5.5
500	1.075 ± 0.068	6.3
Between-days precision ($n = 6$)		
20	0.127 ± 0.023	18.1
40	0.162 ± 0.013	8.0
100	0.275 ± 0.041	14.9
200	0.498 ± 0.039	7.8
500	1.015 ± 0.139	13.6

a minimum of maintenance. The column was regenerated once or twice a month by repacking the top. More than 600 plasma samples could be assayed on the same column.

Validation procedure

Validation of the method was performed prior to the assay of toxicokinetic samples and continued throughout the study. The summaries of within run precision and the between run precision of the method appear in Table I.

The percentage coefficient of variation of peak area ratios was taken as a measure of precision. The percentage coefficient of variation varied randomly over the concentration range. The accuracy of the HPLC method was determined by recalculating the concentration of the calibration plasma samples using the calibration curve (Table II).

Linearity of response was demonstrated by linear regression analysis from concentrations of 20–500 ng/ml ($n = 6$). The least-squares correlation values ranged from 0.9987 to 0.9999 (mean ± S.D. = 0.9993 ± 0.0005, $n = 6$) with slopes between

TABLE II

ACCURACY OF HPLC PROCEDURE FOR RGH-5702

Theoretical concentration (ng/ml)	Peak area ratio	Recalculated concentration
20	0.035 ± 0.006	22.35 ± 7.65
40	0.061 ± 0.011	41.48 ± 3.51
100	0.135 ± 0.015	98.48 ± 4.69
200	0.260 ± 0.023	200.80 ± 9.70
500	0.650 ± 0.033	500.00 ± 2.82

TABLE III

RECOVERY OF RGH-5702 IN DOG PLASMA

Concentration (ng/ml)	Recovery (% mean ± S.D., $n = 5$)	C.V. (%)
20	81.8 ± 9.5	11.6
40	81.0 ± 8.5	10.5
100	82.5 ± 5.7	6.9
200	78.5 ± 4.1	5.2
500	76.2 ± 10.2	13.4
1000	79.3 ± 7.4	9.3
2000	74.6 ± 5.1	6.8
Mean ± S.D.	79.1 ± 2.9	

$1.166 \cdot 10^{-3}$ and $1.360 \cdot 10^{-3}$ (mean ± S.D. = $1.284 \cdot 10^{-3} \pm 0.065 \cdot 10^{-3}$, $n = 6$). We also checked that the extension of the calibration range to 2000 ng/ml did not affect the linearity.

Plasma recovery

The recovery of the assay was determined as the detector response of pure authentic standard solutions compared to the response from extracted plasma calibration samples containing an equivalent amount of RGH-5702. The percentage of recovery of the compound was calculated as:

$$\text{recovery} = \frac{\text{peak area of extr. plasma calibration sample}}{\text{peak area of standard solution}}$$

(An aliquot of stock solution of RGH-5702 was further diluted and dried into vial, reconstituted in eluent then injected.) The results are shown in Table III and the overall recovery of the assay was 79.1 ± 2.9%.

CONCLUSION

A quick and selective liquid chromatographic method for the determination of RGH-5702 in dog plasma has been developed. The quantitation limit for the method is 20 ng/ml with a signal-to-noise ratio of > 5. The HPLC method described in this paper enables the monitoring of RGH-5702 during toxicokinetic and chronic toxicity studies.

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CHROMSYMP. 2351

High-performance liquid chromatographic determination of rifapentine and its metabolite in human plasma by direct injection into a shielded hydrophobic phase column

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ABSTRACT

A simple high-performance liquid chromatographic method for determination of rifapentine, a cyclopentyl semisynthetic analogue of rifamycin belonging to the class of piperazinyl hydrazone derivatives of 3-formylrifamycin SV, and its metabolite, 25-desacetylrifapentine, in human plasma was developed using direct injection of the sample onto a Supelco LC HISEP column. The mean recovery was 100.3% for rifapentine and 99.7% for the metabolite and the precision of the assays was 3% and 7%, respectively. The limit of determination was 0.2 μg/ml and the method was validated for concentrations up to 64 μg/ml for rifapentine and 32 μg/ml for the metabolite. The results correlated well with those of the microbiological assay with *Sarcina lutea* as test organism.

INTRODUCTION

Rifapentine (Fig. 1), an antibiotic currently used in clinical trials, has usually been assayed by a conventional microbiological assay with *Sarcina lutea* as test organism or using a multi-step extraction procedure with different solvents (ethyl acetate,

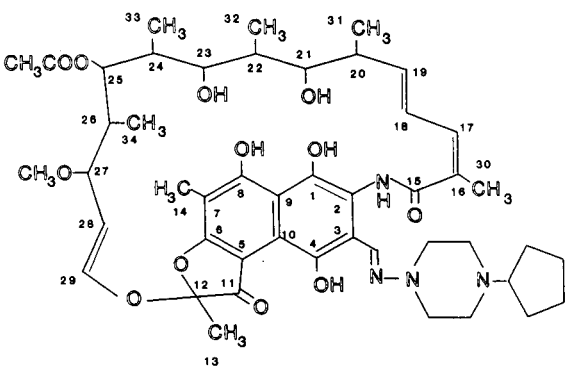

Fig. 1. Structure of rifapentine (MW 877.06).

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acetonitrile, *n*-heptane) followed by high-performance liquid chromatography (HPLC) [1–3].

We have recently developed an HPLC method for the determination of the antibiotic and its major metabolite in human plasma that uses direct injection of biological samples. It has the advantage over the previous HPLC method of eliminating several time-consuming steps, therefore increasing the accuracy, while retaining the advantage over the microbiological assay of permitting the determination, in the same assay, of rifapentine and its main metabolite. The shielded hydrophobic phase column used (Supelcosil LC HISEP) [4–6] represent a new concept for the separation of drugs because it is designed to avoid precipitation of the protein on the chromatographic support.

This paper describes a simple HPLC method for the determination of the level of rifapentine (rifap) and of its major metabolite, 25-desacetylrifapentine (desa), in human plasma that only requires filtration of the sample.

EXPERIMENTAL

Chemicals and reagents

Rifapentine, batch LCR-E/5/85, and 25-desacetylrifapentine, batch C/6/87, were internal standards produced by Lepetit. Analytical-reagent grade 2-(N-morpholino)ethanesulphonic acid (MES) was purchased from Sigma and trifluoroacetic acid and ethanolamine from Carlo Erba. Acetonitrile, methanol and tetrahydrofuran of HPLC grade were purchased from Carlo Erba or Rudi Pont.

Standard solutions

Stock standard solutions of rifap and desa were prepared by dissolution in methanol at a concentration of 2 mg/ml then diluted with distilled water, containing 1 mg/ml of ascorbic acid, to a final concentration of 1 mg/ml.

Spiked samples were prepared by adding increasing amounts of the stock standard solution to human plasma to obtain the following concentrations in μg/ml: sample 1, 0.94 rifap, 0.47 desa; sample 2, 3.78 rifap, 1.88 desa; sample 3, 15.13 rifap, 7.52 desa; and sample 4, 60.54 rifap, 30.08 desa. Each spiked sample was analysed five times on each of three different days and the analytical response obtained are summarized in Table I.

Chromatographic conditions

The HPLC system consisted in two Gilson Model 305 pumps, a dynamic mixer, a Gilson Model 805 manometric module and a Gilson Model 116 UV detector operating at 254 nm, controlled by an IBM PS/2 computer through the Gilson 714 program. Collection and analysis of the detector output were performed with Hewlett-Packard A Series computer using the Hewlett-Packard LDS software. Samples (50 μl) were injected with Gilson ASPEC autosampler onto a guard column (20 × 4.6 mm I.D.) of Supelcosil LC HISEP in series with a Supelcosil LC HISEP column (150 × 4.6 mm I.D.). Gradient elution was performed at room temperature at a flow-rate of 1.4 ml/min. The two mobile phases were (A) 0.0026 *M* MES–acetonitrile (95:5) containing trifluoroacetic acid (1‰) and adjusted to pH 6.5 with ethanolamine and (B) 0.0026 *M* MES–acetonitrile–tetrahydrofuran (7:2:1) containing trifluoroacetic acid

TABLE I

RESULTS FOR FIVE REPLICATES AT FOUR CONCENTRATIONS ANALYSED ON EACH OF THREE SUCCESSIVE DAYS

In the concentration ranges 0.94–60.54 μg/ml for rifapentine and 0.47–30.08 μg/ml for the metabolite the within-day precisions (means of the daily R.S.D. were 1.58%, 1.44%, 1.05% and 1.05% for rifapentine and 5.83%, 3.71%, 2.34% and 0.27% for metabolite, respectively. The between-day precision (R.S.D. of the daily mean) were 2.06%, 0.71%, 0.27% and 0.01% for rifapentine and 2.57%, 2.33%, 0.68% and 2.69% for the metabolite, respectively. The mean accuracies were 100.29% for rifapentine and 99.53% for the metabolite.

Parameter	Rifapentine				25-Desacetylrifapentine			
	Sample (μg/ml) (n = 5)	Day 1	Day 2	Day 3	Sample (μg/ml) (n = 5)	Day 1	Day 2	Day 3
Mean (μg/ml)	0.94	0.96	0.93	0.95	0.47	0.47	0.46	0.45
R.S.D. (%)		1.65	2.23	0.82		6.05	7.03	4.57
Accuracy (%)		102.60	98.58	101.50		100.14	98.48	94.97
Mean (μg/ml)	3.784	3.83	3.78	3.78	1.88	1.83	1.88	1.93
R.S.D. (%)		1.36	1.54	1.40		2.35	2.41	6.39
Accuracy (%)		101.10	99.93	99.80		97.46	99.02	102.46
Mean (μg/ml)	15.13	15.11	15.12	15.19	7.52	7.39	7.66	7.70
R.S.D. (%)		0.79	1.26	1.09		1.48	2.93	2.64
Accuracy (%)		99.87	99.96	100.38		98.08	101.86	102.38
Mean (μg/ml)	60.54	60.49	60.56	60.48	30.08	30.17	30.08	29.88
R.S.D. (%)		1.58	0.93	0.62		0.21	0.38	0.21
Accuracy (%)		99.92	100.04	99.90		100.63	99.62	99.33

(1‰) and adjusted to pH 6.5 with ethanolamine. Elution was carried out with a linear gradient from 30% to 70% B in 24 min after a 4-min run to permit the flow-through of plasma proteins. The column was then conditioned with 30% B for 6 min.

Microbiological assay

The microbiological assay was carried out with the parallel lines design. We used 120-mm diameter Petri dishes filled with 14 ml of antibiotic medium No.1 (pH 6) and inoculated with 0.5% of *Sarcina lutea* ATCC 9341. Three standard solutions were prepared in bovine serum at concentrations of 0.1, 0.2 and 0.4 μg/ml. The samples were preliminarily tested to assess the range of concentrations, and then diluted with bovine serum to obtain concentrations close to those of the standard.

Nine 6-mm diameter wells were made in each of six dishes used for every assay. Two samples were run on each set of dishes against the standard by filling the wells with 0.02 ml of the appropriate solution with an automatic pipette. After overnight incubation at 33°C the diameters of the inhibition zones obtained were measured to the nearest 0.1 mm. When plotted as log(concentration) *vs.* diameter of inhibition zone, the lines for the standard and for the samples were checked for linearity, parallelism and curvature and the potency of the sample was calculated as the mean difference between the two lines. The results were calculated with a computer program according to the statistical approach described in the British Pharmacopoeia 1980 (Appendix XIV A).

Cross-validation

About 450 plasma samples collected from healthy volunteers in a study by the Clinical Research Department of the Lepetit Research Centre, were analysed with the microbiological assay and the results were compared those obtained with the direct injection HPLC method.

RESULTS AND DISCUSSION

HPLC

Direct injection of biological fluids into a liquid chromatograph is advantageous from the standpoints of speed, cost, safety, sample tracking and analytical recovery.

Chromatograms obtained with blank plasma and after spiking it with known amounts of drug and metabolite at concentrations of 10 μg/ml of rifap and 5 μg/ml of desa, and at the limit of determination for desa (0.4 μg/ml rifap and 0.2 μg/ml desa) are shown in Fig. 2. No interference from endogenous substances in the blank plasma sample was observed within the elution range of the analytes.

During a typical overnight run with 24 plasma samples and six spiked plasma standards, there was no change in the retention times of the drugs and a negligible increase in the pressure in the column. In fact, we found that it was sufficient simply to invert the analytical column between runs to restore the original pressure. We decided to filter each sample through a 0.45-μm acetate membrane (Acrodisk; Gelman) to prevent clogging of the guard column filter.

Assay linearity

Statistical evaluation was carried out following the procedure described by Cavenaghi *et al.* [7].

Calibration graphs were obtained using drug-free plasma spiked with known amounts of drug and metabolite. The standard daily responses were collected for each day and cumulated to calculate the respective daily cumulative least-squares linear regression of the peaks area.

Analysis of variance (ANOVA) with $p = 0.05$ was used to confirm the significance of the regression calculated from the respective daily cumulative calibration graphs.

The calculated day-to-day overall least-squares linear regression is reported in Table II.

Precision and accuracy

Table I summarizes the results obtained by the described method for the analysis of spiked standard samples. The mean accuracy ranged from 95.0% to 102.6%, whereas the within-day precision, indicated by the mean of the daily relative standard deviation (R.S.D.), varied from 0.27% to 5.88%. The reproducibility expressed as the between-day precision indicated by the R.S.D. of the daily means ranged from 0.1% to 2.7%.

Cross-validation

The linear regression determined by the method of least squares on the results

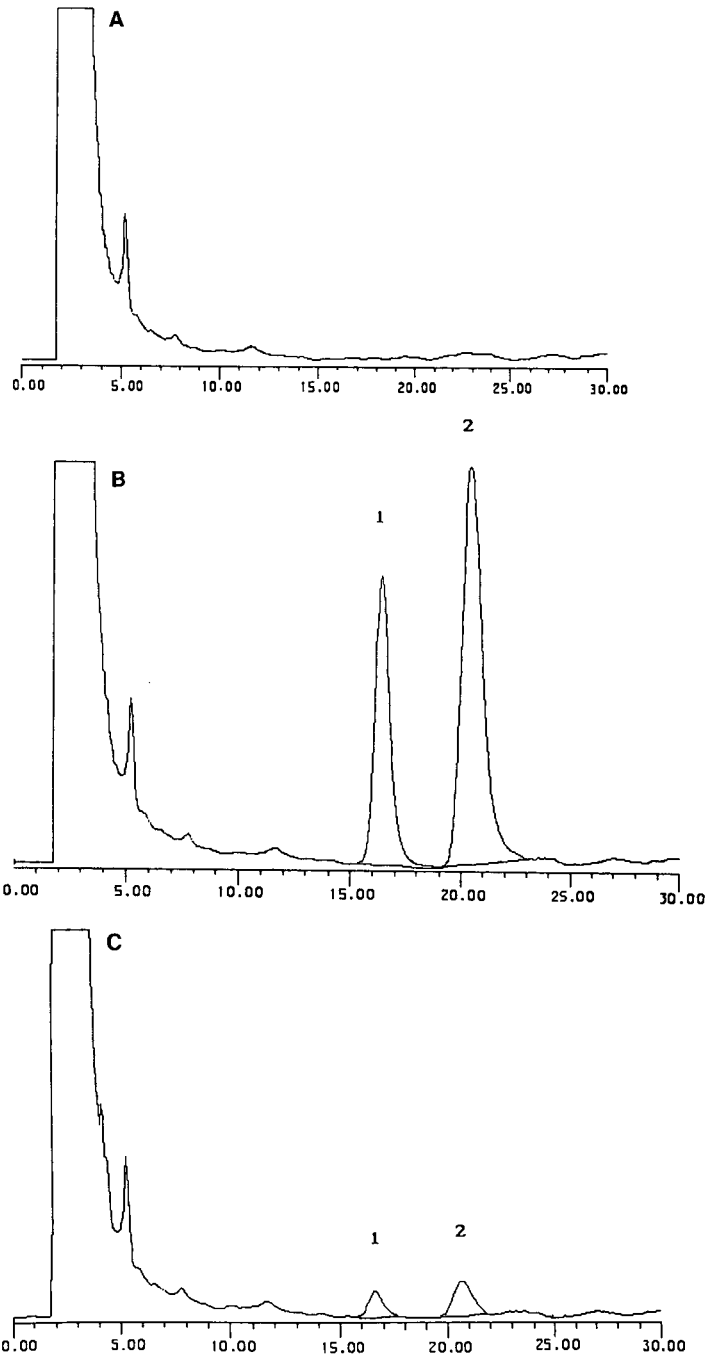

Fig. 2. Chromatograms obtained with (A) drug-free human plasma; (B) plasma spiked with 10 μg/ml of rifap and 5 μg/ml of desa; (C) plasma with drugs at the limit of determination of desa (0.4 μg/ml of rifap, 0.2 μg/ml of desa). Time scale in min.

TABLE II

DAY-TO-DAY CUMULATIVE REGRESSION LINE WITH THE RESPECTIVE ANALYSIS OF VARIANCE

Compound	Day	*n*	Slope	Intercept	Correlation coefficient	*F*-ratio	
						ANOVA	Lack of fit
Rifap	1	5	29693	760	0.99967	49133	0.0125
	2	10(5+5)	29971	512	0.99904	100335	0.0088
	3	15(10+5)	30033	412	0.99972	203199	0.0034
Desa	1	5	30387	−279	0.9999	428698	0.3193
	2	10(5+5)	30337	−164	0.99992	434348	0.1294
	3	15(10+5)	30272	−73	0.99988	454956	0.0145

obtained with the two methods had a slope of 1.006 and the correlation coefficient was 0.9077, indicating that antibiotic concentrations obtained by the HPLC method correlate well with those obtained by microbiological assay.

CONCLUSIONS

The applicability of the direct injection HPLC method to measure rifapentine and its metabolite in plasma was demonstrated. The method has adequate precision and accuracy at plasma levels from 0.94 to 60.54 μg/ml for rifapentine and from 0.47 to 30.08 μg/ml for its metabolite. The good correlation for about 450 samples between the results obtained with the HPLC method and a microbiological assay shows that either method can be used when assessing the total level of antibiotic in human plasma. However, under conditions that may interfere with a microbiological assay (such as the presence of other antibiotics) or when differentiation between the parent compound and the metabolite is necessary, the HPLC method is to be preferred.

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CHROMSYMP. 2157

Micro-liquid chromatography method for the determination of ciclopiroxolamine after pre-column derivatization in topical formulations

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ABSTRACT

Ciclopiroxolamine is a broad-spectrum antimycotic drug. Neither the free acid (ciclopirox) nor its salt with ethanolamine (ciclopiroxolamine) can directly be quantified by liquid chromatography (LC) on both normal and reversed phases. This is due to the chelating function of the N-hydroxypyridone group that interacts strongly with stationary phases. Derivatization by alkylation forms a 1-alkyloxypirydone with regular chromatographic behaviour. A micro-LC method based on an isocratic elution reversed-phase system for quantification of ciclopiroxolamine in topical formulations is described. Chromatography was carried out using an LC Packings fused-silica capillary column (15 cm × 330 μm I.D.; Delta Pak, RP-18, 5 μm, 300 Å) coupled to a Kontron 433 UV capillary detector. Data with respect to the derivatization reaction, recovery, reproducibility and limits of detection of the proposed method are reported and discussed.

INTRODUCTION

Ciclopiroxolamine, the 2-aminoethanol salt of 6-cyclohexyl-1-hydroxy-4-methyl-2(1H)-pyridone (Fig. 1), is a synthetic antifungal agent with a broad spectrum of *in vitro* activity against most pathogenic fungi, including dermatophytes, *Candida albicans* and numerous non-pathogenic fungi [1–4]. Like many other antimycotic drugs, ciclopiroxolamine possesses antibacterial properties. Its activity against *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and relevant *Staphylococcus* and *Streptococcus* species has also been demonstrated [5].

Good penetration through hornified skin layers shows that ciclopiroxolamine is a useful drug in onychomycosis therapy [6]. Its antifungal action differs from other antimycotic drugs, in that ciclopiroxolamine exerts its activity not directly on the

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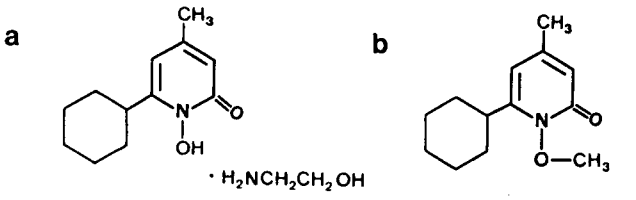

Fig. 1. Chemical structure of (a) ciclopiroxolamine and (b) 1-methoxyciclopiroxolamine

fungal plasma membrane but by intracellular depletion of some essential substrates and/or ions caused by inhibition of their uptake [7].

Ciclopiroxolamine is used, at relatively low concentration (1%), in different pharmaceutical formulations (creams, powders, lotions). Spectrophotometric [8] and high-performance thin-layer chromatography (HPTLC) [9] methods have been proposed for the quality control of various pharmaceutical dosage forms containing ciclopiroxolamine. The spectrophotometric method does not seem sufficiently quantitative when ciclopiroxolamine is incorporated into a complex matrix such as cream. Furthermore it involves preliminary extraction procedures that are laborious and time consuming.

In addition, HPTLC methods lack the sensitivity required for analysis of pharmaceutical preparations: the free acid and its salt with aminoethanol migrate as a uniform spot and severe tailing was also observed.

An alternative approach to quantitative determination of ciclopiroxolamine is liquid chromatography (LC). However, with the use of silica-based material two problems are encountered: irreversible absorption of small amounts of ciclopiroxolamine and tailing peaks. The strong absorption of ciclopiroxolamine onto the packing material observed is presumably due to the chelating function of the N-hydroxipyridone group. This interaction results in non-linear calibration curves. Methylation of the weak acidic N-hydroxyl group of the ciclopiroxolamine gave the 1-methoxy derivative that shows a normal chromatographic behaviour [10].

The present paper describes a micro-LC method based on an isocratic elution reversed-phase system for direct quantitation of the 1-methoxy derivative of ciclopiroxolamine in commercial dosage forms (foam and powder).

EXPERIMENTAL

Reagents

Acetonitrile was of HPLC grade (Fluka, Buchs, Switzerland); distilled water used was purified by a Water I system (Gelman, MI, U.S.A.). All other reagents were of analytical-reagent grade and were used without further purification.

Samples

Pharmaceutical dosage forms, foam and powder, containing 1% ciclopiroxolamine were obtained from Laboratori Delalande (Italy).

Sample preparation: foam. A sample equivalent to about 10 mg of the antimycotic drug was weighed exactly in a 50-ml calibrated flask. For derivatization, 2 ml

of 1 *M* sodium hydroxide solution and 30 μl of methyl iodide were added. After vortex-mixing, the flask was kept in an ice-bath for 10 min. Methyl iodide excess was destroyed by adding 30 μl of 25% ammonium hydroxide solution to the mixture. It was then dissolved and diluted to volume with acetonitrile–water (1:1, v/v). An aliquot of 200 nl was injected into the chromatographic system.

Sample preparation: powder. A sample equivalent to about 2 mg of the antimycotic drug was weighed exactly in a 10-ml PTFE-lined screw-capped glass tube, then 2 ml of acetonitrile–water (1:1, v/v), 1 ml of 1 *M* sodium hydroxide solution and 20 μl of methyl iodide were added. After vortex-mixing, the tube was kept in a ice-bath for 10 min. Methyl iodide excess was destroyed by adding 20 μl of 25% ammonium hydroxide solution to the mixture.

The mixture was vortexed and centrifuged (10 min, 3000 *g*). The liquid layer was carefully transferred into a 50-ml volumetric flask. The solid residue was resuspended in 5 ml of acetonitrile–water (1:1, v/v) and again extracted. The liquid phase was carefully transferred directly into the previous volumetric flask; this was repeated twice. The volume was then adjusted to 50 ml with the extraction mixture. An aliquot of 200 nl was injected into the chromatographic system.

Standard

Ciclopiroxolamine standard of know purity was provided by Sibefat (Milan, Italy) and was used without further purification.

Calibration procedures. The calibration curves of ciclopiroxolamine in foam and powder were constructed from replicate samples over the concentration range 0–12 mg/g. Samples of control (drug free) foam and powder were mixed with appropriate amounts of ciclopiroxolamine standard, weighed exactly on a Cahn model G2 electrobalance, to give concentrations of 0, 2, 4, 6, 8, 10 and 12 mg/g. All the samples were derivatized according to the procedure described above. The peak areas were then compared to those obtained from derivatized ciclopiroxolamine standard solutions with comparable concentrations.

Accuracy

The accuracy of the method was checked by means of recovery experiments carried out on representative pharmaceutical preparations (foam and powder) spiked with known quantities of the drug to give three series of 10 samples with concentrations of 8, 10 or 12 mg of the drug per g of foam and powder.

Apparatus

The LC system consisted of the following components: Carlo Erba (Milan, Italy) Phoenix 20 CU micro-pump; Kontron (Zurich, Switzerland) 433 UV capillary detector, equiped with an ultrasensitive UV flow-cell (total volume 90 nl, optical path length 20 mm); Valco (Houston, TX, U.S.A.) injector Model C14W with a 200-nl internal loop. Peak areas were measured with a Perkin-Elmer (Norwalk, CT, U.S.A.) LCI-100 laboratory computing integrator.

Chromatographic conditions. A Fusica column (LC Packings, Amsterdam, The Netherlands) (15 cm × 330 μm I.D.; Delta Pak, RP-18, 5 μm, 300 Å) was used. The column was connected directly the flow-cell via a small piece of PTFE tubing (tubing kit TF-K1, LC Packings).

The separations reported were achieved under the following conditions: mobile phase, acetonitrile–water (1:1, v/v); flow-rate 10 μl/min; chart speed, 0.5 cm/min; temperature, 20°C; wavelength, 300 nm. The quantity of ciclopiroxolamine was calculated by applying the external standard method.

RESULTS AND DISCUSSION

The irreversible absorption of ciclopiroxolamine on reversed-phase which appears in non-linear response to the quantity injected and the peak area or height, in different sequential injections of the same amount, is assumed to be the result of the chelating properties of the analyte with residual silanol groups on the surface of the stationary phase. In agreement with reported data, methylation of ciclopiroxolamine gave a stable derivative that shows a single non-tailing peak [10].

Derivatization of ciclopiroxolamine by means of reaction with methyl iodide

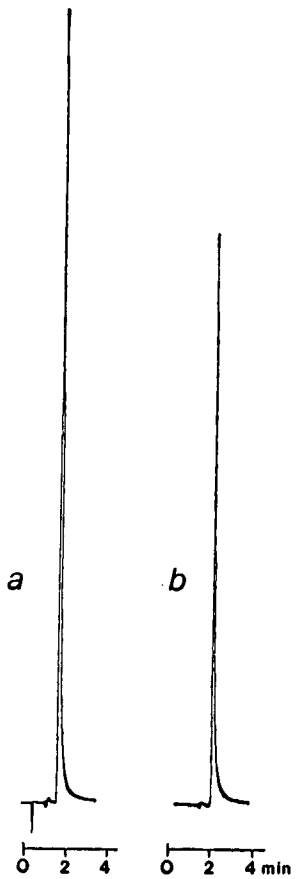

Fig. 2. Chromatograms of ciclopiroxolamine in (a) dermal foam and (b) in topical powder. Column, Fusica (15 cm × 330 μm I.D.; Delta Pak, RP-18, 5 μm, 300 Å); mobile phase, acetonitrile–water (1:1, v/v); flow-rate 10 μl/min; chart speed, 0.5/min; temperature, 20°C; wavelength, 300 nm.

and dimethyl sulphate was investigated. The methyl iodide reagent was selected due to its ability to react more rapidly.

Examples of chromatograms resulting from the LC assay of ciclopiroxolamine in foam and powder are shown in Fig. 2. With the chromatographic solvent system used, the Fusica column was found to give sharp, symmetrical peaks and good selectivity. No interference at the retention time of the analyte peaks due to other active or inactive substances present in the pharmaceutical forms was observed.

The overall precision of the retention time was studied with respect to run-to-run and day-to-day precision. The run-to-run precision for 30 runs within a single day averaged 1.21% relative standard deviation (R.S.D.), while a day-to-day precision over a 4-week period with the same column averaged slightly more, *ca.* 1.97% R.S.D.

The calibration graphs for the measurement of ciclopiroxolamine in foam and powder were constructed by analyzing spiked samples over drug concentration ranges 0–12 mg/g. The response was linear over the concentration range examined for both dosage forms. The linear least-squares regression relationships for ciclopiroxolamine in foam and powder were $y = 1.012\ x + 0.054$ and $y = 1.120\ x + 0.8753$, respectively.

The assay recoveries of ciclopiroxolamine from spiked foam and powder samples were 98.1% with 1.5% R.S.D. and 96.1% with 2.4% R.S.D., respectively. The detection limit of ciclopiroxolamine (signal-to-noise ratio 3) was 10 ng.

In conclusion, the proposed method offers high selectivity and sensitivity for the determination of ciclopiroxolamine in commercial dosage form.

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High-performance liquid chromatographic determination of vinblastine, 4-O-deacetylvinblastine and the potential metabolite 4-O-deacetylvinblastine-3-oic acid in biological fluids

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ABSTRACT

Procedures for the determination of vinblastine (VBL), 4-O-deacetylvinblastine (DVBL) and 4-O-deacetylvinblastine-3-oic acid (DVBLA) in biological samples using high-performance liquid chromatography (HPLC) combined with selective sample clean-up are presented. VBL and DVBL were determined in plasma and urine using ion-exchange normal-phase HPLC with fluorescence detection. The limit of detection was 1 μg/l for both compounds using a 500-μl sample. Successful chromatographic analyses of DVBLA were achieved by using a glass column packed with 5-μm Hypersil ODS and acetonitrile–0.05 *M* phosphate buffer (pH 2.7) (23:77, v/v). Positive identification was supported by the use of diode-array detection. The limit of detection (at 270 nm) was 10 μg/l using 1-ml samples.

INTRODUCTION

Vinblastine (VBL) (Fig. 1) and vincristine (VCR) are naturally occurring vinca alkaloids derived from the periwinkle plant *Catharantus roseus* G. Don and are active in a variety of human neoplastic disorders. It is generally believed that the interaction with microtubular protein is related to the cytotoxic action. In order to improve the therapeutic efficacy and/or to change the antitumour range of this class of com-

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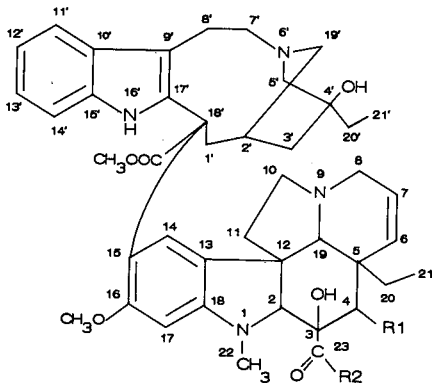

	R1	R2
VBL	$OCOCH_3$	OCH_3
DVBL	OH	OCH_3
DVBLA	OH	OH

Fig. 1. Structures of VBL, DVBL and DVBLA.

pounds, a diverse array of analogues have been developed. Vindesine (VDS) was the first of these semi-synthetic derivatives shown to be clinically useful. Other analogues are currently under clinical or preclinical investigation [1–5].

Several high-performance liquid chromatographic (HPLC) methods have been described for the determination of vinca alkaloids in plasma even at very low concentrations (1 μg/l) [6–10]. Most studies provided data on the pharmacokinetics of the parent vinca alkaloid under investigation. However, these studies were not focused on the bioanalysis of metabolic conversion products. Available information on this subject originates from older papers and is restricted to VBL, VCR and VDS only. Indications for possible metabolic conversion have been obtained with the aid of radioactively labelled drugs but without further structural elucidation [11–13]. Sethi *et al.* [14] provided data on the presence of deformylvincristine in urine from a patient who had received vincristine, and Owellen *et al.* [15] identified 4-deacetylvinblastine (DVBL) in stool and urine from patients receiving VBL.

A knowledge of the metabolic fate of vinblastine and other vinca alkaloids is essential to extend the insight into the disposition of the drug and its metabolites and clinical activity and toxicity. With vinca alkaloids, metabolites may contribute to an important extent to the antitumour activity, *e.g.*, Owellen *et al.* [15] reported DVBL to be an even more active compound than VBL in the *in vitro* test systems that they used. Apart from the 4-acetyl group of the VBL molecule, the 3- and 18′-methyl ester groups might also be susceptible to metabolic conversion to the respective carboxylic acids.

This paper is the first report on the bio-analysis of such a potential metabolic compound, *viz.*, 4-O-deacetylvinblastine-3-oic acid (DVBLA), in biological fluids.

Other theoretical metabolites (*e.g.*, vinblastine-3-oic acid and vinblastine-18-oic acid) are not yet available. Some preliminary data concerning the search for DVBL and DVBLA metabolites in human plasma and urine samples from cancer patients receiving VBL are presented.

EXPERIMENTAL

Reagents

The vinca alkaloids VBL, DVBL, DVBLA and N-(deacetyl-O-4-vinblastoyl-23)-L-ethyltryptophan (vintriptol, VtrpE) were provided by Medgenix (Brussels, Belgium) and VDS was obtained commercially. All other reagents were purchased from Merck (Darmstadt, Germany) and were of analytical-reagent grade, except for acetonitrile and chloroform, which were of HPLC-grade. Water was purified with a Mille-Q system (Millipore–Waters, Milford, MA, U.S.A.).

Instrumentation

The chromatographic analyses were performed using an HPLC system consisting of a Spectroflow SF400 pump, a Model 1000S photodiode-array detector, a Model SF980 fluorescence detector (all from Kratos, Ramsey, NJ, U.S.A.) and an MSI 660 autosampler (Kontron, Basle, Switzerland). Peak recording and integration were done on a WINNER-4 data station (Spectra-Physics, San Jose, CA, U.S.A.). Spectral data analysis was achieved on an IBM-compatible computer provided with Lab Calc software Galactic Industries, Salem, NH, U.S.A.).

Determination of VBL and DVBL

The procedures for the determination of VBL and DVBL were similar to those described previously for the investigational cytotoxic semi-synthetic derivative of VBL, N-(deacetyl-O-4-vinblastoyl-23)-L-ethyl isoleucinate (VileE) [9]. Standards solutions of VBL and DVBL were prepared by diluting stock solutions (1 mg/ml) directly with the appropriate matrix (plasma or urine). Calibration graphs were constructed in the range 1–1000 μg/l for VBL and 1–100 μg/l for DVBL.

A mixture of 500 μl of plasma or urine, 10 μl of internal standard (10 mg/l of VtrpE in acetonitrile) and 2.5 ml of 0.5 *M* phosphate buffer (pH 4.0) was extracted with 5 ml of chloroform in a glass tube. The tubes were mixed for 10 min, followed by centrifugation for 10 min at 2500 *g*. The organic layer was transferred to a clean tube and evaporated to dryness under nitrogen (37°C). The residue was dissolved in 100 μl of acetonitrile and an aliquot of 80 μl was analysed using a stainless-steel column (250 × 2 mm I.D.) packed with 5-μm Spherisorb Si. The mobile phase was acetonitrile–0.01 *M* citrate buffer (pH 3.0) (85:15, v/v) containing 0.01 *M* tetrabutylammonium bromide. The flow-rate was maintained at 0.2 ml/min. Fluorescence detection was used with excitation at 270 nm, the emission being monitored using a 320-nm long-pass filter. Ratios of the peak height of VBL or DVBL to that of the internal standard were used for quantitative calculations.

Determination of DVBLA

In glass tubes fitted with PTFE-covered screw-caps (Renes, Zeist, The Netherlands), 5 ml of 0.5 *M* phosphate buffer (pH 7.5) were added to 1000 μl of plasma. For

urine samples 1 ml of 1.0 *M* phosphate buffer (pH 9.0) was added to 1000 μl of samle. Next 10 μl of internal standard solution (20 mg/l of VDS in acetonitrile) and 5 ml of chloroform were added and the tubes were mixed for 10 min. After centrifugation for 10 min at 2500 *g*, the aqueous layer was discarded and the organic layer was transferred into a polyethylene Pony vial (Packard Instruments, Groningen, The Netherlands). A volume of 1000 μl of 0.05 *M* phosphate buffer (pH 2.5) was added and the vials were gently mixed for 5 min and subsequently centrifuged for 5 min at 2500 *g*. A volume of 700 μl of the aqueous phase was transferred into to a Model 3813 sample vial (Eppendorf, Hamburg, Germany) and lyophilized. The residue was dissolved in 100 μl of acetonitrile–water mixture (20:80).

An aliquot of 50 μl of this sample was analysed using a glass column (100 × 3 mm I.D.) packed with 5-μm Hypersil ODS preceded by a guard column (10 × 3 mm I.D.) packed with pellicular reversed-phase material (Chrompack, Middelburg, The Netherlands). The mobile phase was acetonitrile–0.05 *M* phosphate buffer (pH 2.7) (23:77, v/v). An amount of 2.5 g of tetramethylammonium chloride was added to 1 l of the mobile phase. The flow-rate was maintained at 0.4 ml/min. For quantitative purposes UV detection at 270 nm was used. Ratios of the DVBLA and VDS peak areas were used for quantitative analysis.

Patients' samples

Urine and heparinized blood samples were collected shortly before and *ca.* 1 h after administration of VBL. Plasma was obtained by centrifugation for 5 min at 2500 *g*. Plasma and urine samples were stored at −20°C until analysis within 4 weeks. Patient 1 (male, 40 years old, 70 kg) received intravenously (i.v) 6 mg/m^2 of VBL together with doxorubicin (65 mg/m^2) and bleomycin (10 mg/m^2). Patient 2 (male, 30 years old, 72 kg) also received i.v. 6 mg/m^2 of VBL together with epirubicin (70 mg/m^2), bleomycin (10 mg/m^2) and orally prednisone (40 mg/m^2).

RESULTS

Because solutions of vinca alkaloids in water are not stable, all dilution steps made during the preparation of the calibration graph should be done in plasma or urine. The detection limits of the procedure for VBL and DVBL were 1 μg/l for both compounds and a linear concentration response curve ($r > 0.99$) was obtained up to 1000 μg/l. The recoveries of extra VBL and DVBL added to patients' samples (plasma and urine) did not differ significantly from 100%. Typical within-day relative standard deviations (R.S.D.s) (calculated from the variation found between duplicates) for plasma samples were 6.7% for VBL and 7.3% for DVBL ($n = 70$), and the day-to-day R.S.D.s were 7.8% for VBL and 8.4% for DVBL ($n = 8$). For urine samples the within-day R.S.D.s were 2.5% for VBL and 3.2% for DVBL ($n = 32$) and the day-to-day R.S.D.s were 4.5% for VBL and 4.4% for DVBL ($n = 7$).

HPLC of DVBLA was performed using a Hypersil ODS column in combination with an acidic mobile phase. The peak tailing of VDS was reduced by adding tetramethylammonium chloride to the mobile phase. A linear concentration response curve for DVBLA diluted in acetonitrile–water (20:80) was obtained down to 20 μg/l, which was the minimum detectable concentration (50 μl injected, signal-to-noise ratio = 3:1). However, when DVBLA was diluted in 0.05 *M* phosphate buffer (pH 2.5) in

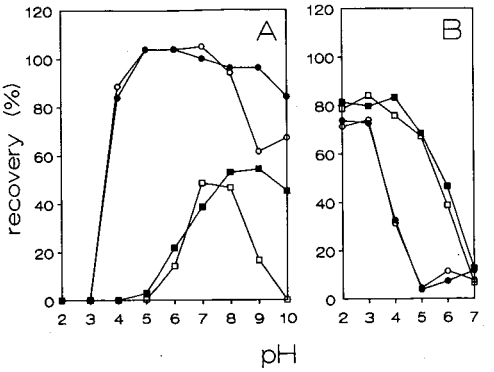

Fig. 2. (A) Recoveries of DVBLA and VDS (1 mg/l each) extracted from plasma or urine. 1000 μl of plasma diluted with 5 ml of 0.5 *M* phosphate buffer or 1000 μl of urine diluted with 1 ml of 1.0 *M* phosphate buffer were extracted with 5 ml of chloroform. The pH of the buffer ranged from 2 to 10. (B) Recoveries of the back-extraction of DVBLA and VDS. Blank plasma and urine samples diluted with phosphate buffer of pH 7.5 and 9, respectively, were extracted with chloroform. The organic phases were spiked with DVBLA and VDS and re-extracted with 0.05 *M* phosphate buffer (pH 2–7). The highest recovery in the aqueous phase was attained at low pH. ○ = VDS, plasma; ● = VDS, urine; □ = DVBLA, plasma; ■ = DVBLA, urine.

Pony vials a detection limit of 30 μg/l was achieved, with a concentration response curve that showed some upward curvature, probably caused by adsorption to the materials used.

Fig. 2 depicts extraction efficiencies of DVBLA and VDS from plasma and urine samples diluted with phosphate buffer. The extraction recoveries depended on the pH of the buffer and the optimum pH was slightly different for plasma and urine.

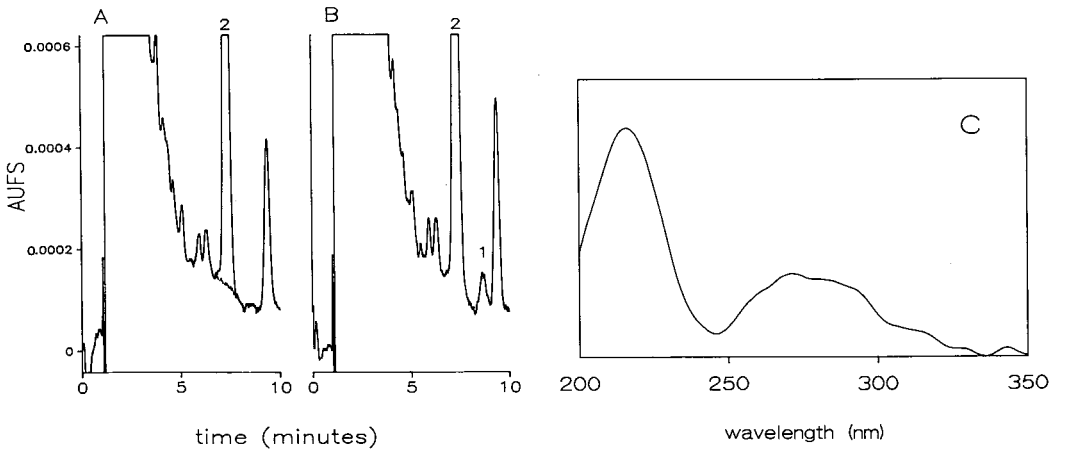

Fig. 3. HPLC of DVBLA. (A) Chromatogram of a blank urine sample [2 = VDS (internal standard; I.S.)]. The broken line represents the chromatogram without any I.S. added. (B) Chromatogram of the same specimen spiked with 20 μg/l of DVBLA. 1 = DVBLA; 2 = VDS (I.S.). (C) UV spectrum of the DVBLA peak from the urine sample spiked with 20 μg/l.

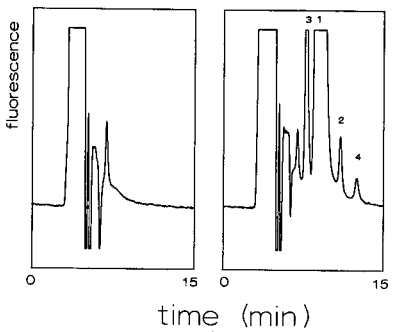

Fig. 4. HPLC of VBL and DVBL in urine. (A) Chromatogram of a urine sample from patient 2 collected shortly before administration of VBL. (B) Chromatogram of a urine.sample from patient 2 collected 1 h after administration of VBL. 1 = VBL; 2 = DVBL; 3 = VtrpE (I.S.); 4 = unknown.

The recoveries, obtained by back-extraction of the compounds from the organic phase into 0.05 *M* phosphate buffer, were optimum at acidic pH (2–3). With these procedures the minimum detectable concentrations in urine and plasma samples were 10 μg/l. The calibration graphs were not linear but showed a slight upward curvature similar to the concentration response curves of DVBLA diluted in 0.05 *M* phosphate buffer (pH 2.5). The within-run R.S.D. was 7.3% (concentration range 20–500 μg/l; $n = 32$) and the run-to-run R.S.D. for a sample spiked with 130 μg/l was 8.0% ($n = 4$).

Fig. 3 shows the chromatograms of a blank urine sample and a sample spiked with DVBLA (final concentration 20 μg/l). Even at these low DVBLA concentrations a useful UV spectrum corresponding to that of the reference compounds was obtained (Fig. 3C).

The plasma concentrations of VBL were 26 and 18 μg/l. No DVBL was detected in plasma. The urine concentrations of VBL were 6900 and 1510 μg/l and those of DVBL were 64 and 11 μg/l (patients 1 and 2, respectively). The chromatographic results for VBL and DVBL in urine samples from patient 2 are shown in Fig. 4. No DVBLA could be detected in either the plasma or urine from both patients.

DISCUSSION

In previous papers we described the determination of vinca alkaloids in plasma and showed that these compounds can be effectively extracted by the use of chloroform [8,9]. Analysis of these extracts using HPLC combined with sensitive and selective detection techniques (fluorescence or electrochemical detection) allowed detection down to 1–2 μg/l levels. After modification of the procedure described for VileE [9], the assay could also be used for the determination of VBL and DVBL. Dilution of samples with phosphate buffer of pH 4.0 permitted the simultaneous determination of VBL and DVBL. VtrpE was used as an internal standard because the extraction recovery at pH 4.0 was higher than that for VileE.

The determination of DVBLA was hampered by its unusual behaviour (extreme peak tailing and non-linearity) under most of the HPLC conditions tested. Only the combination of a Hypersil ODS or μBondapak C_{18} column and a mobile

phase buffered at pH 3.0 yielded useful chromatographic results. However, selective detection techniques could not be used, as the fluorescence and electrochemical activities of DVBLA are negligible under these conditions. UV detection at 270 nm could be used but lacked the selectivity required to analyse samples pretreated by simple one-step extraction. An extra clean-up step was therefore necessary. The pH dependence of the partition coefficient between the aqueous and organic phase allowed the back-extraction of DVBLA in a buffer solution of pH 2.5 with a high recovery. The high recovery of DVBLA in the aqueous phase might indicate that the overall polarity on this compound is determined more by the degree protonation of the nitrogen atoms in the molecule [N-1, pK_a = 3.8; N-6′ and N-9, pK_a = 6.6 (80°C)] [16] than by the dissociation state of the carboxylic acid (pK_a = 4.2). Samples thus obtained contained no interfering compounds even if detection in the low-UV range (210 nm) was employed, enabling reliable UV spectra to be acquired. For quantitative purposes UV detection at 270 nm was preferred, combining sufficient sensitivity with optimum selectivity.

With the proposed method we were able to show that DVBLA is probably not a major metabolic product from VBL, as no detectable concentrations were found in plasma or urine samples from patients receiving VBL. However, the number of patients and the sampling times used were very limited. Results concerning the possible formation of DVBLA as a metabolite of vinblastine or other experimental vinca alkaloids, currently under investigation, will be presented in future papers.

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CHROMSYMP. 2133

Two-dimensional high-performance liquid chromatography at low ng/ml levels of the anti-proliferative agent B859-35 in serum with automated sample clean-up, solid-phase trapping and ultraviolet detection

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ABSTRACT

An automated non-chiral high-performance liquid chromatographic method is described for the determination of the new anti-proliferative agent B859-35 in serum. This method employs sample clean-up of 1 ml of biofluid by liquid–solid extraction with the AASP (Advanced Automatic Sample Preparation) system. First separation is achieved on a LiChrospher-60-RP-Select-B column. A fraction of this eluate is then collected by solid-phase trapping. Thereafter, the final chromatogram is developed on a narrow-bore Hypersl-CPS column and quantified with ultraviolet detection at 230 nm. The limit of quantitation of the assay is 250 ng/ml. Linearity was proven in the range 0.25–100 ng/ml. Typical figures for precision at these concentrations are 7.4 and 3.3%, and for accuracy 8.0 and 1.3%, respectively. An application of this method to the study of pharmacokinetics of B859-35 in serum samples of cancer patients is given.

INTRODUCTION

(−)-3-[3-(4,4-Diphenyl-1-piperidinyl)propyl]-5-methyl-1,4-dihydro-2,6-dimethyl-4(*R*)-3-nitrophenyl)pyridine-3,5-dicarboxylate-hydrochloride (B859-35) is the (−)-enantiomer of a dihydropyridine derivative (Fig. 1) with pronounced anti-proliferative activity in *in vitro* as well as in *in vivo* experiments [1,2].

The optical purity of this compound is > 99.5%. There is no evidence so far for a lack of *in vitro* or *in vivo* configurational stability of this enantiomer and, therefore,

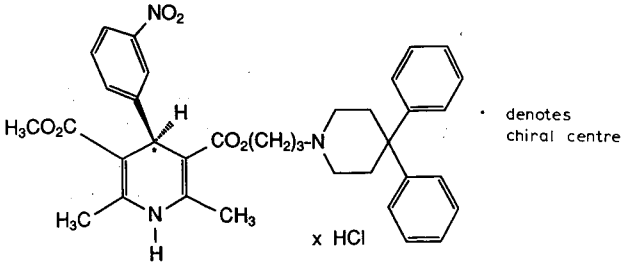

Fig. 1. Structure of B859-35.

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the development of a non-chiral assay seemed to be appropriate. The compound has only negligible cardiovascular effects when compared with the racemate or the (+)-enantiomer [3,4] and its therapeutic usefulness in cancer patients is under clinical evaluation at present.

Due to the very lipophilic nature of B859-35, the volume of distribution (derived from preclinical experiments) is, at 20–40 l/kg in animals, rather high. Consequently most of the administered compound is not in the blood but is distributed into various tissues within the body. Thus for determination of B859-35 concentrations in serum a very sensitive assay has had to be developed.

B859-35 has no functional moieties which can be derivatized to allow for the introduction of a chromophor or fluorophor which will enhance the sensitivity of the assay, nor does electrochemical oxidation contribute to detector response as can be done with other dihydropyridine derivatives [5]. Furthermore it is not sufficiently volatile for gas chromatography.

Direct injection of large volumes (200 μl) of serum or plasma into a fully automated pre-column switching system is used in our laboratory as a bioassay for many compounds [6,7]. To lower the detection limit for B859-35, however, use of 1 ml of serum was necessary. The typical capacity of the pre-column for liquid–solid extraction in the automated pre-column switching device [6] is, however, limited to a minimum of *ca.* 15–20 ml of total injected volume of serum (*e.g.* 75–150 injections of 200 μl each). Therefore, the AASP (Advanced Automatic Sample Preparation) system was introduced for liquid–solid extraction using 1 ml of serum. A new cartridge is used for each serum specimen in this system.

It was the aim of this application to combine the semi on-line technique of liquid–solid extraction provided by the AASP system with a second step of purification of the analyte using two-dimensional chromatography for further removal of matrix components interfering with the peak of interest [8].

EXPERIMENTAL

Chemicals

Standard compound was synthesized by Byk Gulden Lomberg (Konstanz, Germany) and was > 99% chemically pure by high-performance liquid chromatography (HPLC) analysis. Optical purity was > 99.5% as determined by nuclear magnetic resonance (NMR) spectroscopy with shift reagents. All reagents were supplied by E. Merck (Darmstadt, Germany) and were analytical grade for buffer substances and gradient grade for solvents.

Preparation of drug solutions and serum external standards

Stock standard solution of B859-35 at a concentration of 100 μg/ml was prepared by dissolving 10 mg in 100 ml of dimethylsulphoxide (DMSO). DMSO was used to prevent adsorption of B859-35 on glass and synthetic materials. The DMSO stock solution was further diluted to appropriate concentrations with standardized human serum for the external standard calibration and for checking the linearity range.

Apparatus (Fig. 2)

Sample clean-up was performed with the AASP station (Varian, Darmstadt, Germany). This system for liquid–solid extraction of the compound of interest from the serum matrix consisted of an AASP cassette holder for off-line treatment, loading and purging the cartridges, and an AASP sampler to insert the individual cartridges into the mobile phase of the HPLC system. RP-2 extraction cartridges (ICT Handels GmbH, Frankfurt, Germany) were used in this clean-up step.

The first analytical column was a LiChrospher-60-RP-Select-B cartridge (5 μm,

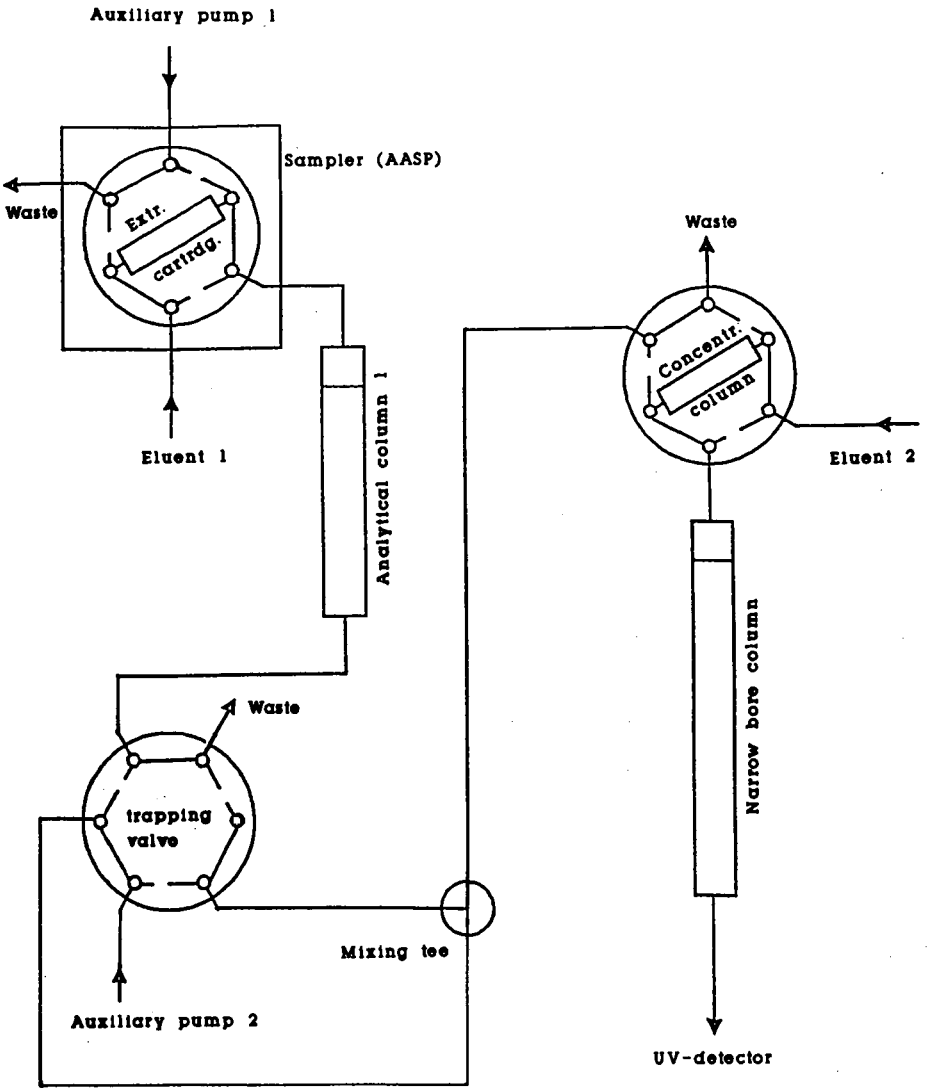

Fig. 2. Column-switching arrangement schematically showing the AASP device, the two analytical columns and the concentration column which precedes the narrow-bore column.

125 × 4 mm I.D.) (E. Merck). A broad fraction of the eluate from the HPLC system was collected by solid-phase trapping using a six-port Rheodyne 7010 pneumatic valve. Further dilution of the trapped fraction to decrease elution strength was necessary using a HKP-11 pump (Labotron Messtechnik, Munich, Germany) to achieve sufficient retention on a RP-8 Nucleosil (5 μm particle size) concentration column (10 × 2 mm I.D.) (Grom, Ammerbuch, Germany). This small column was connected via a Rheodyne 7010 pneumatic valve to the narrow-bore column (200 × 2 mm I.D.) filled with 5 μm Hypersil-CPS (Grom).

A HP 1090 liquid chromatography unit with a built-in filter photometer detector (FPD) and thermostatically controlled column compartment was used (Hewlett Packard, Waldbronn, Germany). Integration was performed by means of the Laboratory Data System HP 3357 (Hewlett Packard).

Sample preparation, column switching, phases

In the first off-line clean-up step liquid–solid extraction of serum samples [6] was carried out by means of AASP cassettes containing ten cartridges filled with alkyl-C_2 bonded phases. The cartridges were placed in a AASP holder which passed liquid through the cassettes by means of pressurized nitrogen and each cartridge was washed with 1 ml of methanol and 1 ml of water for cleaning and wetting. The washed cartridges were conditioned with 2 ml of a 40 m*M* aqueous solution of tetrabutylammonium hydroxide (TBAH) for ionpair formation with the acidic compound B859-35. A 1-ml specimen of serum (centrifuged for 10 min at *ca.* 5000 *g* to remove solid particles) was diluted in a separate step with 800 μl water, then 200 μl of formic acid (100%) were added to this diluted serum to decrease plasma protein binding. This solution was then passed through the conditioned cartridges in 1-ml steps followed by a last wash step with 1 ml of 5 m*M* sodium phosphate buffer pH 7.0. Cassettes with cartridges loaded by this procedure were kept at 4°C until filling of the AASP injection device, thus serving as autosamplers.

Immediately prior to automatic insertion into the analytical mobile phase eluent, each cartridge was purged in the AASP sampler with 500 μl of 5 m*M* sodium phosphate buffer pH 7.0. The connection time of each AASP cartridge to the analytical column was 2 min. Thereafter, the first chromatogram was developed on a LiChrospher-60-RP-Select-B column. The isocratic eluent was premixed and consisted of 5 m*M* sodium phosphate buffer pH 7.0-acetonitrile (35:65, v/v) at a flow-rate of 1.0 ml/min. Collection of a fraction of the eluate by solid-phase trapping started 1 min before the retention time of the compound of interest on the first column. (Retention time is determined before running the first analysis with a standard sample at a concentration of *ca.* 500 ng/ml.) The duration of the trapping window was set to 4 min while the column eluate was transferred to the concentration column for peak compression following on-line dilution with an aqueous 20 m*M* sodium perchlorate buffer pH 2.0, at a flow-rate of 2.2 ml/min to decrease elution strength in order to achieve sufficient retention on the second column [7].

Immediately after the completion of eluate collection, the concentration column with the trapped fraction was connected to the narrow-bore column serving as the second analytical column for 2 min to develop the final chromatogram. A premixed mobile phase of 5 m*M* sodium phosphate buffer pH 7.0 with acetonitrile (32:68, v/v) was used. The flow-rate of this column was adjusted to 0.3 ml/min. The

peak of interest was detected with the FPD at a wavelength of 230 nm. All columns were placed in the HP 1090 column compartment with the temperature adjusted to 40°C.

Before use all eluents were purged with helium to remove dissolved gases. Water for the preparation of solutions and buffers was purified on a Milli-Q-purification unit (Millipore, Eschborn, Germany).

Preparation of standard curves

Standards for the calibration curve were made by spiking control serum at concentrations of 0.25, 0.5, 1.0, 5.0, 25, 50 and 100 ng/ml. Each spiked serum standard was injected ten times. Standard curves were constructed by plotting the chromatographic peak areas against the known concentrations of B859-35 in serum. Individual calibration curves were calculated by ordinary least-squares regression analysis, and the concentration of B859-35 quantified by relating the respective peak area to the appropriate curve.

To calculate loss of compound by solid-phase trapping and DMSO, solutions at concentrations of 0.5 and 25 ng/ml were injected directly on the narrow-bore column and peak areas were compared with those areas at the same concentrations which resulted from chromatography of spiked serum samples through the complete solid-phase system. Each standard was injected five times.

RESULTS AND DISCUSSION

Linearity, precision, accuracy, and recovery

Linearity was examined at concentrations within the range 0.25–100 ng/ml (Table I). Each standard was injected ten times. The calibration curve thus derived was described by the equation: $y = 0.9978\,x + 0.2678$ with a coefficient of correlation $r = 0.9993$. Accuracy was + 8% for the lower limit of quantitation and 1.3% for 100 ng/ml. Values for precision [coefficient of variation (C.V.)] at these concentrations were 7.4 and 3.3%, respectively. Recovery was checked with five subsequent injections each at 0.5 and 25 ng/ml. Values of 47.0 and 53.8%, respectively, showed similar

TABLE I

PRECISION, ACCURACY AND LINEARITY FOR B859-35 (SPIKED SERUM SAMPLES)

Standard regression line: $y = 0.9978x + 0.2678$; $r = 0.9993$.

Set point (ng/ml)	Actual value (Mean ± S.D., $n = 10$) (ng/ml)	Precision (%)	Accuracy (%)
0.25	0.27 ± 0.02	7.4	+ 8.0
0.50	0.58 ± 0.04	6.9	+ 13.8
1.00	1.18 ± 0.05	4.2	+ 18.0
5.00	5.15 ± 0.21	4.1	+ 3.0
25.00	25.10 ± 0.90	3.6	+ 0.2
50.00	46.70 ± 2.90	6.4	− 6.7
100.00	101.30 ± 3.34	3.3	+ 1.3

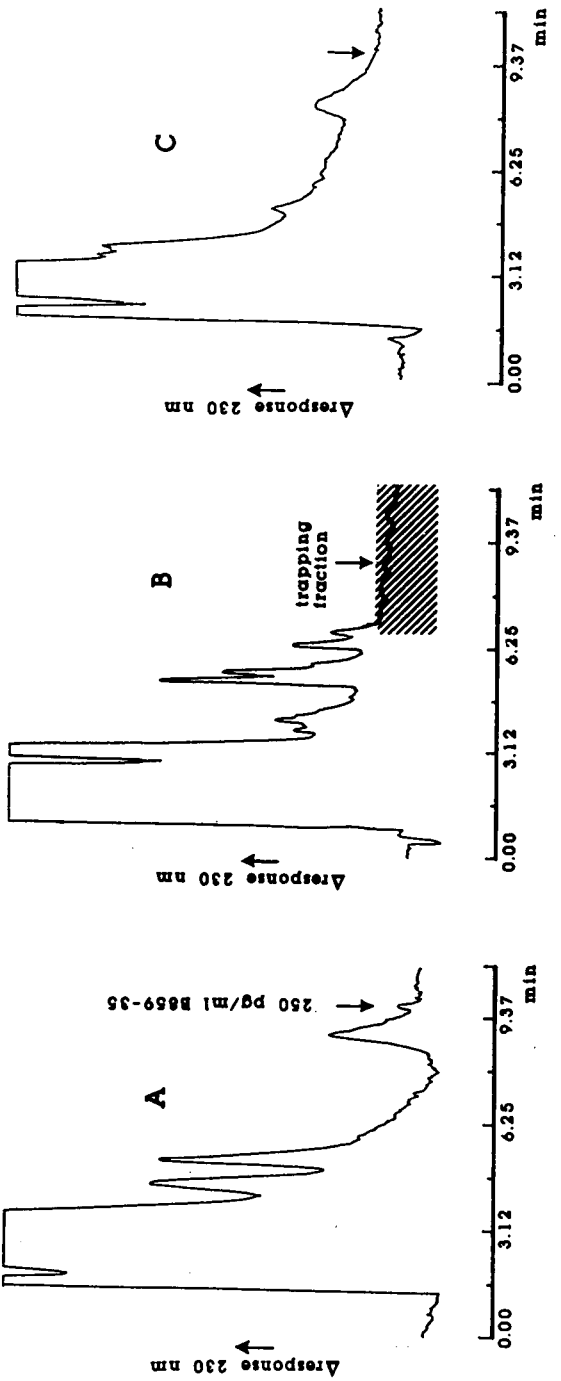

Fig. 3. Final chromatograms of a blank (C) and spiked (250 pg/ml) serum sample (A) eluting from the second (narrow-bore) column. Chromatogram (B) shows elution with the trapping fraction used for concentration and transfer to the second column.

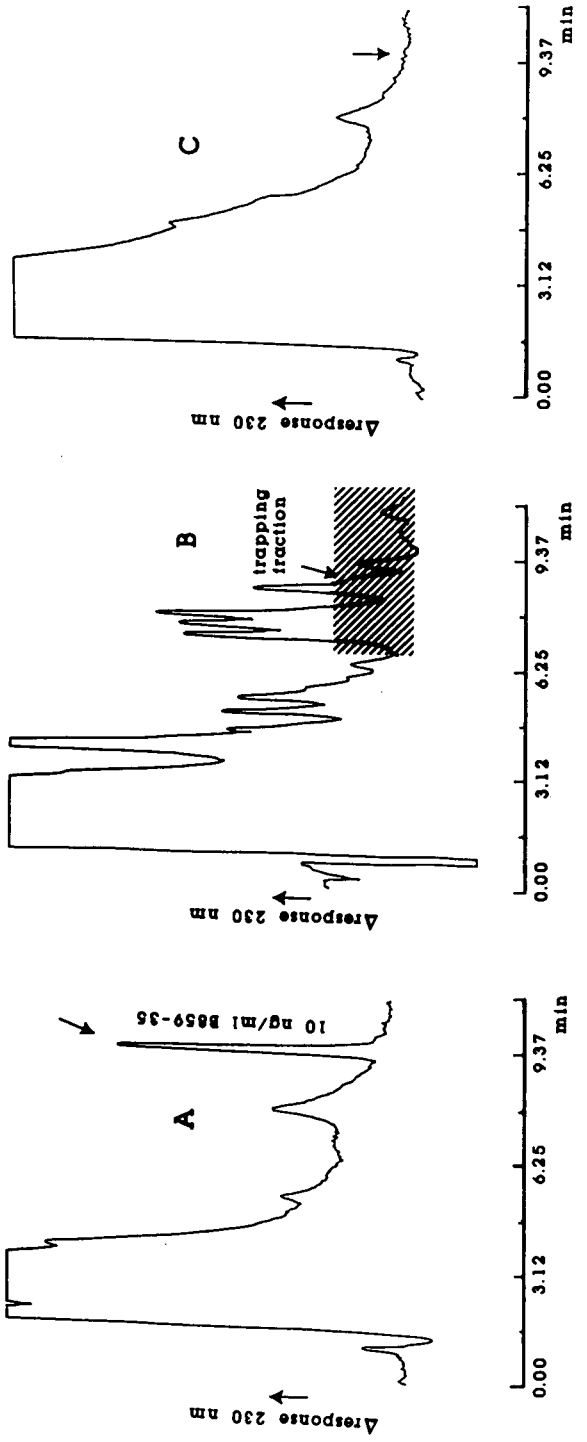

Fig. 4. Chromatograms of a serum sample from a cancer patient 2 h after administration of a 100-mg capsule of B859-35. The serum concentration at this time point is assayed for 10 ng/ml. Chromatograms eluting from the first (B) and second (A) column are given, as well as a pre-dose sample of the same patient (C) eluting from the second column.

recoveries at low and at high concentrations of B859-35 in serum. In these figures a mean recovery of 82% on the AASP cartridge (determined by [^{14}C]B859-35; data not shown) contributes quite considerably to the overall relatively low, but constant, recovery over the entire assay system.

Chromatograms

Fig. 3 shows typical chromatograms of spiked serum standards and Fig. 4 chromatograms of samples obtained from a cancer patient. Comparison of chromatograms from the first and second columns demonstrates clearly the removal of the residual interfering serum matrix achieved by the two-dimensional technique. Therefore, analysis of as much as 1 ml of serum on a narrow-bore column is feasible in order to achieve a high assay sensitivity.

Application

Fig. 5 demonstrates the applicability of the assay. Individual serum B859-35 profiles from four male cancer patients after receiving a 100-mg capsule of B859-35 are shown.

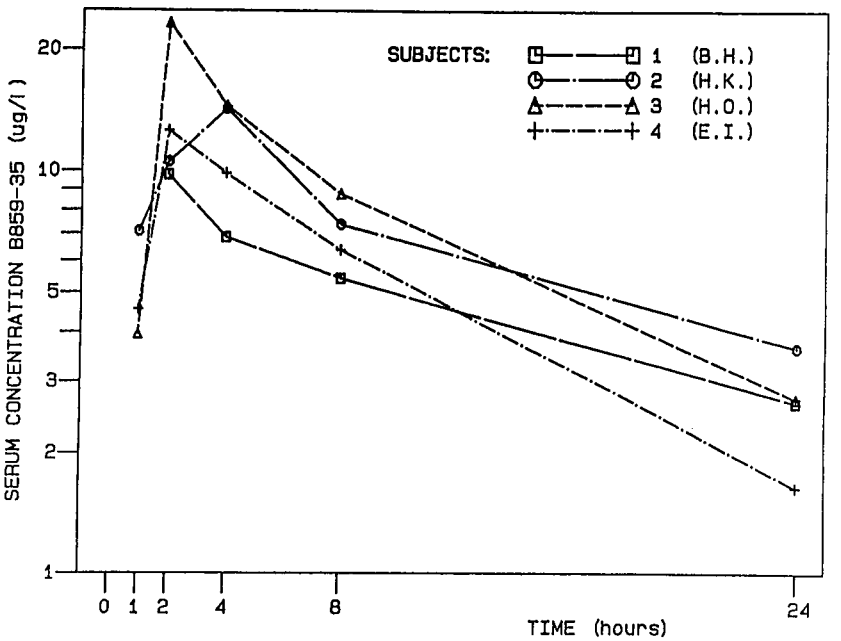

Fig. 5. Individual concentration–time profiles for B859-35 in serum of four cancer patients following single oral administration of a 100-mg capsule of B859-35.

CONCLUSIONS

As the mode of detection for B859-35 is restricted to UV absorption, optimum removal of the interfering plasma matrix was essential to decrease both the detection

limit and the lower limit of quantitation of this bioassay. The automated technique presented enables quantitation of the new anti-proliferative drug B859-35 at very low ng/ml values using 1 ml of serum or plasma. Sample clean-up was obtained in a first off-line step by liquid-solid extraction with the AASP system. Further separation of the interfering matrix was achieved by on-line collection of a fraction with the peak of interest by solid-phase trapping followed by on-line transfer to a second analytical column. For the latter, a narrow-bore column was used to achieve the best sensitivity. Concentration of the collected fraction was therefore a prerequisite for development of the final chromatogram. Up to 40 samples can be handled in one day and for the following series of samples only the concentration column has to be replaced.

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CHROMSYMP. 2134

Sensitive determination of the phosphodiesterase III/IV inhibitor zardaverine in human serum by direct sample injection, automated precolumn clean-up and high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method is described for the determination of the phosphodiesterase III/IV inhibitor zardaverine in serum by using fully automated clean-up of large-volume serum samples on a semi-preparative-scale precolumn followed by chromatography on two analytical columns operated with two different solvent systems. The switching of the analytical columns provides the necessary specificity and sufficient sensitivity for UV detection is obtained by the sample volume. The method was shown to give nearly quantitative recovery, allowing the use of external standard quantification. Good precision and linearity within the concentration range 1–50 ng/ml could be demonstrated. The method is suitable for routine measurements in support of kinetic studies of zardaverine in man.

INTRODUCTION

Zardaverine [6-(4-difluormethoxy-3-methoxyphenyl)-3(2*H*)-pyridazinone] is a new phosphodiesterase (PDE) III/IV inhibitor (Fig. 1), exhibiting bronchospasmolytic and anti-inflammatory activity as shown both *in vitro* and in animal models [1,2]. To

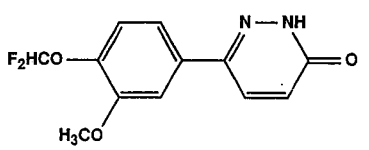

Fig. 1. Structure of zardaverine.

establish its potential therapeutic use for treatment of asthma in man, zardaverine is currently being evaluated in clinical phase II. In order to support human kinetic studies, a sensitive method had to be developed for the determination of the drug in serum down to the low ng/ml range.

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EXPERIMENTAL

Materials

Hypersil ODS (5 μm), LiChroprep RP-2 (25–40 μm), empty precolumns (30 × 8 mm I.D., Hyperchrom) and empty analytical columns (125 × 4.6 mm I.D., Hyperchrom) were supplied by Grom (Amerbuch, Germany). The internal length of the precolumn was shortened to 10 mm by insertion of a 20 × 8 mm PTFE rod with a bore of 0.25 mm. All column endings were closed by metal sieves rather than by frits.

Zardaverine and the marker compound [6-(3,4-methylenedioxyphenyl)-3(2*H*)-pyridazinone] were synthesized at Byk Gulden (Konstanz, Germany) and were >99% pure as tested by high-performance liquid chromatography (HPLC). Methanol and acetonitrile were obtained from E. Merck (Darmstadt, Germany) and were of LiChrosolv quality. Reagents were purchased from E. Merck and were of analytical-reagent grade, unless stated otherwise.

Apparatus

The first part of the HPLC system consisted of a Gynkotek (Munich, Germany) Model 300 high-precision pump. The auxiliary pump was a Gilson Model 302 (Abimed, Langenfeld, Germany), which was connected to a Gilson Model 231-401 auto-sampling injector (Abimed), the latter consisting of a sample controller, a Model 231 sample injector and a Model 401 diluter. Column switching of the precolumn to the first analytical column was done by means of a pneumatic Rheodyne Model 7010A six-port valve (Latek, Heidelberg, Germany), which was controlled by an external contact of the sample controller. A valve of the same type was used for the transfer of the heart-cut from the first analytical column to the second chromatographic system, which consisted of a Hewlett-Packard (Waldbronn, Germany) Model HP1084B liquid chromatograph. The second switching valve was contributed by the column switching option 79823A of the HP1084B system. The effluents from the two analytical columns were monitored at 253 nm by two Kratos (Karlsruhe, Germany) Spectroflow 757 UV detectors and the signals were recorded on a Hitachi Model D-2000 chromato-integrator (E. Merck) for the first detector and the HP79850B integrator of the HP1084B for the second detector. Integration of chromatograms was performed by means of a Hewlett-Packard HP 3357 laboratory data system.

The analytical columns were packed by using an HPLC column packing unit (Latek, Heidelberg, Germany). Precolumns were dry-filled.

Columns, eluents and column switching

The precolumn was filled with LiChroprep RP-2 (25–40 μm). Both analytical columns contained Hypersil ODS (5 μm). The serum samples were transfered to the precolumn by a stream of 10 m*M* aqueous ammonium phosphate (PH 7) at a flow-rate of 2.0 ml/min. After a flushing period of 10 min to remove matrix components, the precolumn was connected with the first analytical column via the switching valve for 2 min, after which the columns were disconnected again.

The mobile phases for the analytical columns consisted of a mixture of 10 m*M* aqueous ammonium phosphate (pH 7) and either methanol [60:40 (v/v), column 1] or acetonitrile [75:25 (v/v), column 2]. The flow-rate for both analytical columns was set at 1.0 ml/min and both were operated at 40°C. The heart-cut from the first analytical column extended from 17 min until 20 min after injection.

Preparation of aqueous stock solutions and serum external standards

A 10-mg amount of zardaverine or the marker compound was dissolved in 1 ml of 1 *M* sodium hydroxide and 3 ml of methanol by warming. The solutions were made up to 100 ml (zardaverine) or 10 ml (marker compound) with doubly distilled water to provide the stock solutions. The stock solution of the marker compound was further diluted with water to a concentration of 5 μg/ml. For the preparation of spiked serum standards, each 1.3 ml of human serum was mixed with an equal volume of the appropriately diluted aqueous stock solution of zardaverine and 20 μl of the diluted solution of the marker compound were added, the concentration of the marker thus being 77 ng/ml.

Serum samples (1.3 ml) from kinetic studies were diluted 1:1 with water and, after addition of 20 μl of the solution of the marker compound, a total of 2000 μl of the diluted sample was injected.

Precision, day-to-day variation, accuracy and recovery

The precision of the assay was tested by tenfold injections of spiked serum standards at concentrations of 2, 20 and 50 ng/ml. These data were also used to calculate the accuracy as the percentage difference between the expected and the measured values. The day-to-day variation was calculated from the peak areas of spiked serum standards at concentrations of 5, 20 and 50 ng/ml. One batch of spiked standards was produced at each concentration and frozen at −20°C as aliquots, which subsequently were thawed and analysed ($n = 10$ per concentration) within several kinetic experiments over a period of 16 weeks.

The recovery of zardaverine on the precolumn was determined by comparison of the mean peak areas after injection of spiked serum standards on the precolumn with the mean peak areas after direct injection of zardaverine dissolved in eluent 2 on the second analytical column. This procedure ensures that potential losses in performing the heart-cutting are also considered. The recovery was determined at concentrations of 2, 20 and 50 ng/ml after fourfold injections.

Calibration

The calibration line for zardaverine was constructed from the mean (± S.D.) peak areas obtained after fourfold injection of spiked serum standards at concentrations of 1, 2, 5, 10, 35 and 50 ng/ml.

RESULTS AND DISCUSSION

Zardaverine was determined by direct injection of serum onto a precolumn with fully automated sample clean-up in combination with a column-switching method [3,4]. As the serum concentrations of zardaverine in man are in the low ng/ml range, the specificity and sensitivity of the HPLC assay had to be enhanced by transfering a heart-cut from the first analytical column to a second column (Fig. 2). The gain in selectivity on the second column was achieved by changing the organic modifier from acetonitrile to methanol while keeping the stationary phase on both columns the same. The heart-cut window (width 3 min) was checked for each chromatogram by means of the marker compound, which is eluted from the first analytical column 1 min before the start of the heart-cutting.

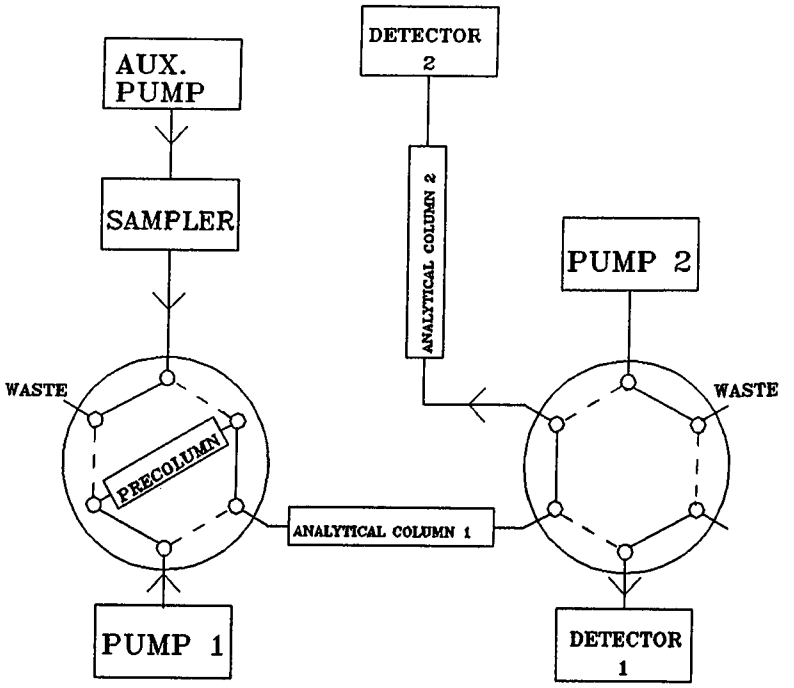

Fig. 2. Scheme of column switching.

The sensitivity required for the low concentrations of zardaverine was achieved by injecting 2 ml of 1:1 diluted serum onto the precolumn. In order to compensate for the heavy sample load, an I.D. of 8 mm was chosen for the precolumn, which thus allowed up to 35 serum samples (= 35 ml) to be processed before replacement of the precolumn was considered necessary. In order to increase the lifetime of the first analytical column, the precolumn was thoroughly flushed for 10 min after sample injection.

The connection time of the precolumn to the first analytical column was optimized by stepwise reduction until the peak area of zardaverine started to decrease. An optimum, yet safe, connection time was found to be 3 min. Optimization of the connection time was essential as a gradual pressure build-up on the precolumn tended to cause a slight decrease in the flow-rate of pump 1 with a consequent shift of the zardaverine peak away from the centre of the heart-cut window. A short connection time of the precolumn obviously would limit this effect. The cycle time for one analysis could be restricted to 32 min, the zardaverine peak eluting at 28 min.

Fig. 3 shows chromatograms of a blank serum sample (B), a serum sample spiked with 20 ng/ml of zardaverine (C) and a serum sample obtained from a volunteer 0.25 h after oral administration of 6 mg of zardaverine (D). These chromatograms were recorded by detector 2 on the second analytical column. Chromatogram A shows the signal of detector 1, the heart-cut region appearing as a plateau, as the detector is bypassed within this time period. Also visible in this plot is the peak of the marker compound.

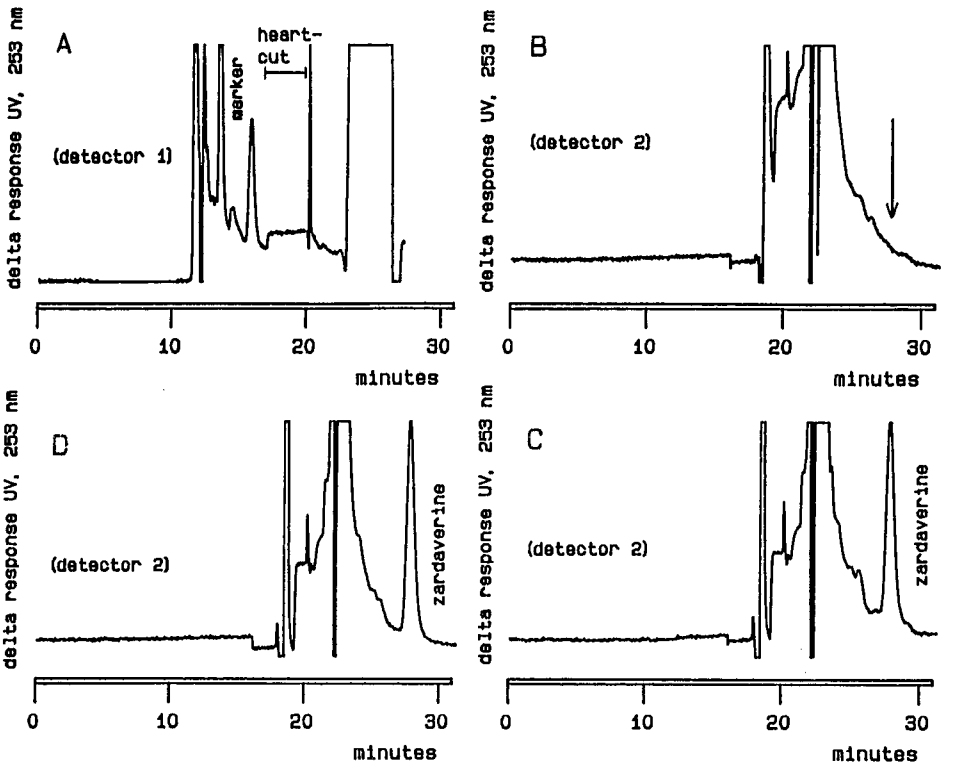

Fig. 3. HPLC after injection of serum samples. Injection volume, 2 ml. (A) Spiked serum standard, trace from detector 1 showing the heart-cut region and the marker compound; (B) blank serum; (C) serum spiked with 20 ng/ml of zardaverine; (D) serum from a human volunteer 0.25 h after oral administration of 6 mg of zardaverine.

Linearity of the assay was tested from 1 to 50 ng/ml, the limit of quantification thus being 1 ng/ml. The regression line obtained from the linearity plot (28 measurements) obeyed the equation $y = 1.04x - 0.84$, the correlation coefficient being 0.9995. The accuracy was between −20% and 0.6% for the concentration range between 1–50 ng/ml (Table I). The relative standard deviation (R.S.D., $n = 10$) for the within-day precision at 2, 20 and 50 ng/ml were 11.8, 2.1 and 1.1%, respectively. The

TABLE I

PRECISION (R.S.D.), ACCURACY AND RECOVERY OF THE ZARDAVERINE ASSAY

Mean concentration ($n = 10$) (ng/ml)		R.S.D. (%)		Accuracy (%)	Recovery (%) ($n = 4$)
Taken	Found	Within-day	Day-to-day		
2	1.6	11.8	–	−20.0	83.6
5	–	–	13.7	–	–
20	19.7	2.1	4.7	− 1.5	98.3
50	52.0	1.2	4.3	− 4.0	101.2

R.S.D.s for the day-to-day variation at concentrations of 5, 20 and 50 ng/ml over a period of 16 weeks were 13.7, 4.7 and 4.3%, respectively. As the peak areas of zardaverine (all standards being from the same batch) did not decrease noticeably, zardaverine in serum is stable at −20°C over this time period. The recoveries at 2, 20 and 50 ng/ml were 84, 98 and 101%, respectively, and may thus be considered to be quantitative, allowing the use of the external standard method for quantification.

CONCLUSIONS

Using automated precolumn clean-up, followed by chromatography on two analytical columns, the HPLC assay allows the rapid, sensitive and accurate determination of zardaverine in serum at concentrations down to 1 ng/ml without any sample pretreatment except for the addition of a marker compound to the serum sample and a 1:1 dilution step with water. The method is suitable for routine measurement, in particular for the characterization of the kinetics of the drug in man.

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CHROMSYMP. 2308

Supercritical fluid chromatography in the routine stability control of antipruritic preparations

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ABSTRACT

A recently developed system for supercritical fluid chromatography (SFC), based on independent flow and pressure control and suitable for packed and capillary columns, was tested on a routine level for the reliable, accurate and precise determination of active pharmaceutical substances in stability control. Only packed columns were used for this analysis. The chromatographic figures of merit and the validation data of the active substance alone and in two different dosage forms (accuracy, 98.8–99.2%; precision, 0.6%; linearity of response, 0.998–0.999) are comparable with the former liquid chromatographic methods. Economical (reduction of analysis time, fewer experimental steps and less sample pre-separation) and ecological (carbon dioxide instead of organic solvents) advantages make SFC an attractive alternative to liquid chromatography in the determination of crotamiton.

INTRODUCTION

In the last ten years liquid chromatography (LC) has become a very important analytical technique in pharmaceutical analytical laboratories [1], where it fulfills many of the needs for reliable, accurate and precise analysis [2]. Compounds of different polarities can be separated in one step by solvent programming if the absorption of the mobile phase does not cause a high baseline slope at low wavelengths (decreased sensitivity). However, an additional equilibration time is required to re-establish the initial conditions.

When the dosage forms are analysed, the LC column must be protected from the deposition of excipients by pre-separation of the active substance.

In contrast to liquids, supercritical fluids have physical properties which give a better mass transfer on the column [3]. As a consequence of this the column reaches equilibrium faster in supercritical fluid chromatography (SFC) than in LC after a mobile phase change [4]. Gradients (pressure, density, or modifier) can be used even at 210 nm without high baseline slopes [ultraviolet (UV) transparency of carbon dioxide, low modifier content]. As a result of the small amount of organic modifier used in the mobile phase, SFC can drastically reduce the consumption of organic solvents in the analysis.

The first-generation SFC instruments were based on syringe pump, capillary columns, flame ionization detection (FID) and pure carbon dioxide as the mobile

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phase [5]. However, the solvation power of carbon dioxide is not sufficient for the elution of very polar or ionic compounds, including many pharmaceutical preparations.

To extend the range of optimum separation conditions and chromatographable substances, special instrumental requirements must be fulfilled [gradients, pressure, density, or (and) modifier; columns, packed (PSFC) or open tubular capillary (CSFC); mobile phase, pure or on-line mixed] [6–10]. With such a second-generation instrument, SFC can be used on a routine basis even for highly polar compounds. This has not yet been shown in detail; the previous problems with polar compounds were cited as a major disadvantage of SFC [11].

On the basis of the author's experience, the advantages of SFC are discussed with respect to the quantitative analysis of different dosage forms of crotamiton, a compound used for the relief of itching.

EXPERIMENTAL

All experimental procedures and evaluation of data were conducted under the Good Laboratory Practice (GLP) guidelines.

Instrumentation

Fig. 1 shows a schematic diagram of a second-generation SFC system suitable for use with PSFC (A) or CSFC (B) [7–10].

A piston pump back-pressure regulator system [9] separates the mobile phase flow-rate and pressure regulation, which leads to the independent control of flow and pressure without any additional calculations [6,12–15].

The SFC system was based on commercial LC hardware (Gilson: two piston pumps 305 (1,2), manometric module 805 (3), mixer 811 (4), sample injector 231 (5)

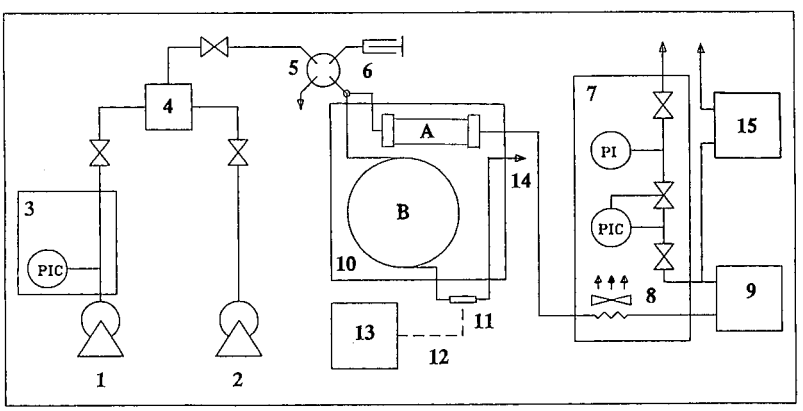

Fig. 1. Second-generation SFC system for PSFC (A) and CSFC (B) columns [7–10]. (1) Carbon dioxide pump, (2) modifier pump, (3) manometric module, (4) mixing chamber/dampener, (5) injection valve, (6) split for capillary column, (7) back-pressure control unit, (8) heat exchanger, (9) UV–visible detector, (10) column oven with PSFC (A) or CSFC (B) columns, (11) capillary UV–visible cell, (12) optical fibers, (13) UV–visible spectrophotometer, (14) heated restrictor–FID, (15) ELSD. (PI) Pressure indicator; (PIC) pressure indicator controller.

(valve Rheodyne 7413 with 1-μl internal loop or 7010 with 5-μl external loop) with diluter 401 combined with a laboratory-made back-pressure controle unit (7) described elsewhere [9]). A bourdon tube (coiled stainless-steel capillary, 2 m × 1/8 in. O.D., 1.8 mm I.D.) acted as an additional pulse dampener. It was placed between the mixer and the injection valve and was mounted in the unit containing the mixing chamber (4), where the temperature was >40°C [8].

A UV–visible absorption detector (9) (Spectra-Physics, Spectra-FOCUS) with an SFC high-pressure cell (volume, 250 nl; path length, 2 mm) was placed between heat exchanger (8) and pressure control unit (7). In certain cases a modified cell with a lower noise level at high flow-rates (volume, 1.6 μl; path length, 2 mm) was used.

An evaporative light scattering detector (ELSD) with a commercial SFC interface (s.e.d.e.r.e., SEDEX 45) was placed after the UV–visible detector to monitor the elution of the excipients in the crotamiton cream dosage form. In this instance the pressure control unit (7) was not used.

The LC instrumentation (Spectra-Physics, SP 8100) used for comparison was equipped with a 20-μl injection loop and UV–visible absorption detector (Spectra-Physics, Spectra-100).

A PS/2 computer (IBM) with a chromatographic software package (Spectra-Physics, LABNET) was used for data collection. Data evaluation was carried out with REPORT MANAGER and the SFC method validation with VAL (programs for Ciba-Geigy).

Columns

The chromatographic columns for SFC (Stagroma) were 100 and 250 × 2 mm, respectively, each equipped with a 20 × 2 mm pre-column and packed with 3-μm particles. Either Spherisorb CN or Nucleosil Si was used, depending on the application.

For LC a 125 × 4.6 mm column packed with Spherisorb Si 80 5-μm particles was used.

Although the dimensions and the particle size were different, the SFC and LC column types show sufficient and comparable resolution for the crotamiton analysis.

Chemicals

For SFC 99.90% grade carbon dioxide (Carbagas) was used as the primary mobile phase component and methanol (Fluka, HPLC grade) as a modifier. Trifluoroacetic acid and ammonium acetate (Merck, spectroscopy and p.a. grade, respectively) in methanol were used as binary modifiers for the chromatography of the crotamiton lotion dosage form.

Compound **1** (numbers refer to peaks in Figs. 2–4) is a possible by-product, 2-phenylethanol (**2**) and sorbic acid (**3**) are preservatives used in the lotion dosage form, **5** is a real by-product, *cis*-crotamiton (**4**) and *trans*-crotamiton (**6**) are together the active substance and **9** is a possible degradation product of the active substance.

The active substance crotamiton is also used in combination with hydrocortisone. Propylparaben and methylparaben (**7** and **8**) are preservatives contained in the cream dosage form, **10** is a possible by-product of hydrocortisone, **11** is a possible degradation product of hydrocortisone and **12** is the active substance hydrocortisone.

The placebo of all three dosage forms used contains the following excipients

without any active substance: (1) crotamiton cream preparation, Lanette O, glyceryl stearate, propylene glycol, liquid paraffin, isopropyl myristate, Myrj 52, water; (2) crotamiton lotion preparation, Lanette N, glyceryl stearate, propylene glycol, Cetomacrogol 1000, citric acid, Eutanol G, perfume creme 45, wool fat, water; (3) crotamiton–hydrocortisone cream preparation, stearyl alcohol, white petroleum, propylene glycol, perfume creme 45, water.

Solutions were prepared in tetrahydrofuran (THF) (Merck, chromatography grade) with the following concentrations of reference compounds: solution A (crotamiton), 2 ng/μl **1**, 1 μg/μl **4–6** and 5 ng/μl **9**; solution B (crotamiton 10% cream), same as solution A plus 10 μg/μl of the appropriate placebo cream; solution C (crotamiton 10% lotion), same as solution A plus 100 ng/μl, **2**, 7 ng/μl **3** and 10 μg/μl placebo lotion; solution D (crotamiton–hydrocortisone), 800 ng/μl **1**, 1.2 μg/μl **5**, 20 μg/μl **4–6**, 100 ng/μl **7** and **8**, 900 ng/μl **9**, 500 ng/μl **10**, 900 ng/μl **11**, and 10 μg/μl **12**. Only solution D must be cleared by filtration before injecting on to the SFC column.

For LC hexane and ethanol (Merck, HPLC grade) were used as the mobile phase. All LC reference solutions were prepared in the mobile phase with the following concentration of reference compounds: solution E (crotamiton), 0.2 ng/μl **1**, 119 ng/μl **4–6** and 0.5 ng/μl **9**.

RESULTS AND DISCUSSION

Method development

For the chromatographic method development the UVabsorption of the compound and its polarity affect the choice of detection mode (UV–visible spectrometry or evaporative light scattering [16,17], or FID), mobile phase polarity (carbon dioxide or carbon dioxide with modifier) and type and polarity of the column. Preference should be given to PSFC. Owing to the higher capacity, lower detection limits and shorter retention times. Compounds which cannot be eluted in their original form can sometimes be made suitable for SFC by derivatisation.

The most polar conditions for initial testing are: high modifier concentration (*e.g.* carbon dioxide + 25% methanol), high density (high pressure, 350–400 bar and low temperature, 40–60°C) and short, rather non-polar columns. Further selectivity can then be achieved through on-line (pressure density, modifier flow, temperature) and off-line (acids/bases in methanol, modifier change, longer and more polar columns) chromatographic variations.

Applications

The different polarities of the compounds present in the preparations mean that two to four chromatographic methods are required to detect each active substance and its corresponding by-products, degradation products and preservations. As a result of the greater analysis speed of SFC, the usefulness of SFC in comparison to conventional LC has been evaluated. PSFC with UV–visible detection is the method of choice.

Crotamiton

cis-Crotamiton (**4**) and *trans*-crotamiton (**6**) and their by-product (**5**) cannot be separated by reversed-phase LC, but can be separated with normal-phase LC as

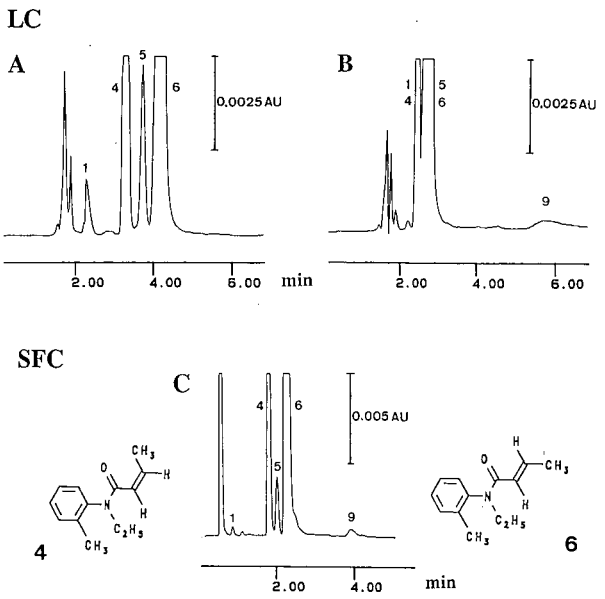

Fig. 2. Chromatograms obtained by (A and B) LC and (C) SFC of *cis*- (**4**) and *trans*-crotamiton (**6**). LC conditions (A and B): column, Si 80, 5 μm, 125 × 4.6 mm; mobile phase, hexane–ethanol (A = 94:6; B = 91:9); flow-rate, 0.9 ml/min; detection, UV absorption at 210 nm; sample, solution E. SFC conditions (C): column, CN. 3 μm, 270 × 2 mm; pressure, 125 (iso)bar; flow-rates, carbon dioxide 1.9 ml/min, methanol 0.05 ml/min; oven temperature, 100°C; detection, UV absorption at 210 nm; SFC cell volume, 1.6 μl; injection loop volume, 5 μl; sample solution A. For other compounds see under Experimental.

shown in Fig. 2A. The possible degradation compound (**9**) elutes very late and is too broad to be quantified. A second LC method using a higher-polarity mobile phase gives a better peak shape for this compound (**9**) as a result of faster elution (Fig. 2B). A combination of both isocratic methods by solvent programming is not usual in normal-phase LC.

With one simple SFC method all five of the important compounds for the analysis of crotamiton are separated under isobaric and isocratic conditions in less than 5 min (Fig. 2C) in the same elution order as in normal-phase LC. The chroma-

TABLE I

CHROMATOGRAPHIC FIGURES OF MERIT FROM THE *trans*-CROTAMITON RESPONSE PEAK AREA (NUMBER OF SAMPLES >20).

Parameter	LC	SFC
Response (% R.S.D.)	0.93	0.44
Retention time (% R.S.D.)	0.19	0.21
Tailing factor[a]	1.47	1.20
Resolution to neighbouring peaks	3.71	1.93
Proportionality of response	0.987	1.016

[a] Tailing factor, see Ref. 18.

TABLE II

LINEARITY, PRECISION AND ACCURACY OF THE SFC METHOD VALIDATION FOR CROTAMITON.

(A) Active substance; (B) active substance in cream; (C) active substance in lotion [19,20].

Statistic	A	B	C
Linearity	0.9998	0.9979	0.9998
Precision (% R.S.D.)	0.6	0.6	0.6
Accuracy (% recovery)	99.2	98.8	99.2

tographic figures of merit (Table I) for the LC and SFC analysis are comparable (tailing factor [18]). Data from the SFC method validation (Table II) are similar to the data from the LC validations.

For the method validation fifteen weighed samples with different concentrations of the active substance, alone or spiked into the appropriate placebo, were analysed (double injection). Linearity, precision and accuracy were calculated from the peak areas (Table II) [19,20].

The linearity of the detector response was measured with five samples of the active substance (50, 75, 100, 125 and 150%; the 100% value is given by the working procedure) and is expressed by the correlation coefficient r.

The precision is related to the variation of ten samples of the active substance alone or spiked into the appropriate placebo [75% (**2**), 100% (**6**) and 125% (**2**)] and is given as percentage relative standard deviation (RSD) based on the peak area relative to 1.0 mg.

The accuracy compares the weight of the sample with the weight found by the linearity curve. Six weighed samples of the active substance, either alone or spiked into the appropriate placebo [75% (**2**), 100% (**2**) or 125% (**2**)], were measured. The result is expressed as an averaged percentage recovery.

Crotamiton and hydrocortisone

A preparation containing crotamiton and hydrocortisone can also be analysed by a single SFC method using a modifier gradient (Fig. 3A). In addition to the five compounds of the crotamiton analysis, the preservatives (**7** and **8**) and the possible by-products and decomposition products (**10** and **11**) are separated from the active substance hydrocortisone (**12**). Additional pressure programming reduces the analysis time by about 0.5 min (Fig. 3B). Prior to the development of this method for stability testing of this preparation, four separate chromatographic methods were required.

Crotamiton dosage forms

To avoid precipitation on the column, a pre-separation of the active substance from the excipients (see under Experimental) is necessary when using the LC-methods.

For SFC the dosage forms (crotamiton cream and lotion) can be diluted in THF and injected directly into the SFC system without any pre-separation (Fig. 4A and C). The THF solution of the crotamiton–hydrocortisone cream must be cleared

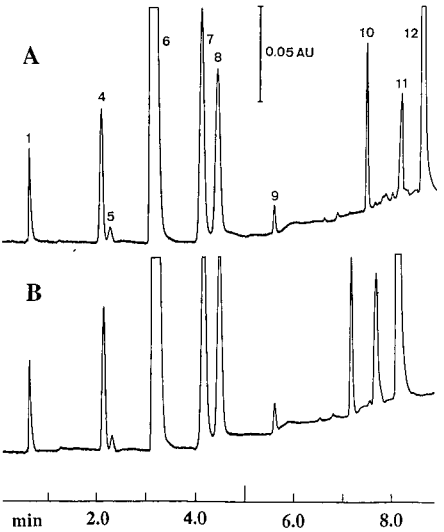

Fig. 3. SFC of crotamiton (**4** and **6**) and hydrocortisone (**12**) with (A) modifier gradient only or (B) modifier and pressure gradient. Conditions: column, Si and CN coupled, each 3 μm, 120 and 100 × 2 mm, respectively; pressure, 125 (iso)bar; flow-rates, carbon dioxide 2.0 ml/min, methanol 0.02 ml/min; oven temperature, 65°C; detection, UV absorption at 254 nm; SFC cell volume, 250 nl; injection loop, 1 μl. Gradients: (A) modifier, 3.5 min isocratic, 0.02 ml/min in 4 min linearly to 0.3 ml/min, 2.5 min isocratic, 0.3 ml/min; (B) modifier (see A) and pressure gradient, 4 min 125 (iso)bar, in 4 min linearly to 250 bar, 2 min 250 (iso)bar. For other compounds, see under Experimental.

by filtration. The elution of the excipients, which does not affect the quantitative UV absorption, is detected by a subsequent ELSD (Fig. 4B). This double detection gives information about the UV active and non-active compounds in just one chromatographic run.

The 10% crotamiton lotion (Fig. 4C) contains two additional preservatives (**2** and **3**) to that of the cream formulation (Fig. 4A). The use of trifluoroacetic acid as an additional binary modifier in the methanol modifier helps to elute the sorbic acid (**3**), but it prevents a secondary amine (**1**) from eluting. Mixing trifluoroacetic acid and ammonium acetate in the methanol modifier gives a satisfactory resolution for both compounds, the base (**1**) and acid (**3**), without any effect on the separation of the other important compounds.

The advantage of these two volatile additives, compared to others such as potassium heptanesulphonate, dimethyloctylamine and tributyamine [21], is their potential usefulness for SFC–ELSD and SFC–mass spectrometry (MS). SFC–MS is especially important for the identification of possible by-products and/or degradation products of active substances in pharmaceuticals.

In general, using SFC rather than LC as a method for the stability control of crotamiton results in both economical (time) and ecological (safety) advantages.

The economical advantages are the reduction of analysis time, fewer chromatographic methods and less sample pre-separation, which reduces the temporal expense of the former LC methods by a factor 3–4. The ecological advantages are the substitution of organic solvents with non-toxic and non-flammable carbon dioxide,

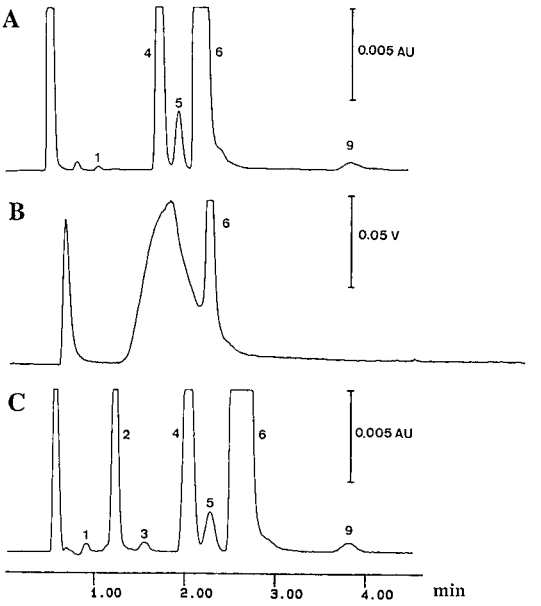

Fig. 4. SFC of crotamiton (**4** and **6**) in different dosage forms (A) cream (UV); (B) cream (ELSD); (C): lotion (UV). Conditions: column, CN, 3 μm, 270 × 2 mm; pressure, 125 (iso)bar; flow-rates, carbon dioxide 1.9 ml/min, methanol 0.05 ml/min; oven temperature, 100°C; detection, (A) UV 210 nm, SFC cell volume 1.6 μl, (B) ELSD 45°C; injection loop, 5 μl. (A and B) Modifier, neat methanol, sample solution B; (C) modifier, 0.035% trifluoroacetic acid and 0.035% ammonium acetate in methanol, sample solution C. For other UV active and non-active compounds see under Experimental.

which increases the laboratory safety and reduces environmental pollution (in this work, less than one tenth of the conventional amount of organic solvent is used).

As a result of these investigations SFC can be used as an alternative method for stability testing. It is suggested that a breakthrough in the use of SFC as a routine chromatographic technique in the laboratory and process control will be seen with the commercial production of second-generation instruments.

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CHROMSYMP. 2298

High-performance liquid chromatographic evaluation of Med 15 and its metabolites Med 5 and tolmetin in rat plasma

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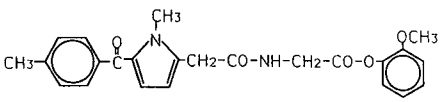
ABSTRACT

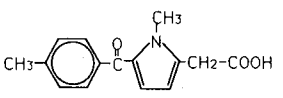
A simple and reliable high-performance liquid chromatographic method is described for the quantitative analysis of the new non-steroidal anti-inflammatory agent Med 15 and its metabolites Med 5 and tolmetin in rat plasma. After selective extraction the three analytes and an internal standard (*p*-phenylphenol) were separated on a reversed-phase Ultrasphere 5 μm column using potassium dihydrogenphosphate (0.05 *M*)–acetonitrile (52:48) (pH 4.7) as the mobile phase. The analytes were detected at 313 nm; the sensitivity of the method proved to be 0.05 μg/ml for all three compounds. The method has been applied to investigate Med 15 pharmacokinetics in rats.

INTRODUCTION

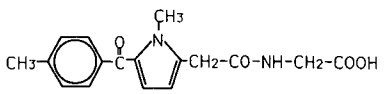
Med 15 is a new non-steroidal anti-inflammatory agent (NSAIA) in which tolmetin is linked to glycine through an amide bond, and the glycine moiety is in turn linked to guaiacol via an ester bond (Fig. 1).



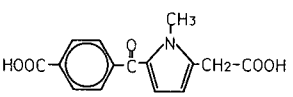
MED 15



TOLMETIN



MED 5



MCPA

Fig. 1. Molecular structures of Med 15, Med 5, tolmetin and MCPA.

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Oral administration of acidic NSAIAs produces typical gastrointestinal side-effects, mainly in the gastric mucosa [1–3]. Many attemps have been made to develop NSAIAs with an improved gastrointestinal tolerance. Esterification of acidic NSAIAs suppresses gastrotoxicity without impairing anti-inflammatory activity [4]. In a previous pharmacological investigation, Med 15 showed sustained anti-inflammatory activity with a particularly good gastrointestinal tolerability [5,6]. This paper describes a reversed-phase high-performance liquid chromatographic (HPLC) method with UV detection for simultaneous determination of Med 15 and its hydrolysis products, namely Med 5 and tolmetin (Fig. 1) using *p*-phenylphenol as internal standard.

EXPERIMENTAL

Chemicals and reagents

Med 15 and Med 5 were supplied by Sigma-Tau (Pomezia, Rome, Italy), and tolmetin and *p*-phenylphenol (internal standard, I.S.) were obtained from Sigma (St. Louis, MO, USA). All other chemicals used were of reagent grade; water was purified with a Milli-Q reagent-grade system. Standard solutions of Med 15, Med 5, tolmetin and the I.S. were prepared in acetonitrile and stored at 4°C.

Animals and treatment

Fasted male Sprague–Dawley rats (Charles River, Como, Italy) (average weight 200 g) were administered a single 82 mg/kg oral dose of Med 15 in a 0.5% carboxymethylcellulose sodium salt suspension. At various times after administration the animals (four per group) were sacrified. Heparinized blood samples were immediately centrifuged and the resulting plasma was separated and stored at −20°C until analysis. Animals were fed 3 h after drug administration.

Chromatographic conditions

The chromatographic system consisted of a Model L-6200 pump (Merck-Hitachi, Darmstadt, Germany), a Model 7125 sample injector (Rheodyne, Cotati, CA, USA) equipped with a 20-μl loop, and a Model L-4200 variable-wavelength UV detector (Merck-Hitachi) operated at 313 nm.

Separation was carried out on a 150 mm × 4.6 mm I.D. Ultrasphere ODS 5 μm column (Beckman, San Ramon, CA, USA) with a guard column (40 mm × 4.6 mm I.D. Perisorb RP-18, 30–40 μm particle size) at room temperature. A Merck-Hitachi D-2500 integrator was used to record chromatograms and calculate the peak heights of the analytes.

The isocratic mobile phase consisted of a 48:52 (v/v) mixture of acetonitrile and potassium dihydrogenphosphate (0.05 *M*) adjusted with phosphoric acid 8.5% (v/v) to a final pH of 4.7. A gradient flow-rate was used to eliminate long retention time of MED 15. It was 0.5 ml/min during the first 5 min, then increased to 0.7 ml/min and after 10 min increased to 1.0 ml/min; Fig. 2 shows the detailed profile of this flow gradient.

An unknown peak present in plasma samples of rats treated with Med 15 (Fig. 2D, MCPA) was identified with a Carlo Erba HRGC 5160 gas chromatograph (Carlo Erba, Milan, Italy) connected to a Finnigan MAT 8222 mass spectrometer with

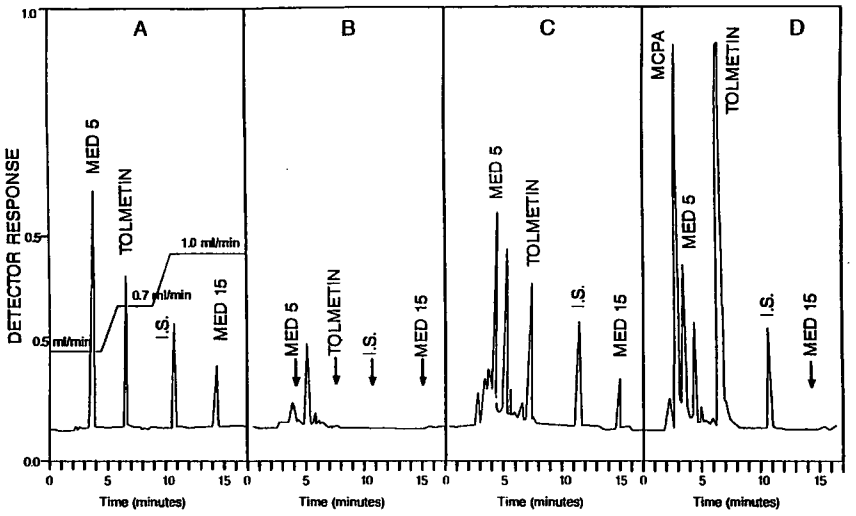

Fig. 2. Typical HPLC chromatograms of (A) analytes as authentic standards, (B) drug-free extracted plasma, (C) analytes added to drug-free plasma, (D) 1 h plasma sample of a rat treated orally with Med 15.

double-focus geometry, equipped with both electron impact and chemical ionization modes (Finnigan MAT, San José, CA, USA). Gas chromatographic analysis was performed with a HP 101 column (15m × 0.32 mm I.D.) (Hewlett-Packard, Palo Alto, CA, USA). The column temperature was programmed from 60°C to 150°C at 25°C/min, and from 150°C to 280°C at 15°C/min, then maintained at 280°C for 5 min. The helium (carrier gas) pressure was 0.25 kg/cm^2.

Extraction procedure

A 1-ml volume of rat plasma was acidified (90 μl of 1.0 *M* HCl), and 20 μl of the I.S. solution (1.0 mg/ml) were added and vortex-mixed for 10 s. The samples were extracted with 6 ml of benzene–*tert.*-butyl alcohol (90:10, v/v) by shaking for 20 min on an automatic shaker. After centrifugation (2000 *g* for 15 min), the organic layer was transferred to a conical tube and evaporated to dryness at 40°C, under a gentle stream of air. The residue was reconstituted with 0.2 ml of mobile phase, sonicated for few seconds and injected into the HPLC column.

Recovery and calibration curves

Percentage recoveries of Med 15, Med 5 and tolmetin from plasma were assessed by comparing the peak-height ratio (drug to I.S.) obtained after extracting known amounts of analyte (0.05–2 μg/ml range) with that obtained when identical amounts of the working standard were dispensed without extraction. Calibration curves were constructed by adding known amounts (0.05–2 μg/ml) of Med 15, Med 5 and tolmetin, to plasma of untreated animals and processing the samples as decribed above. Values of the peak-height ratio (drug to I.S.) for Med 15, Med 5 and tolmetin were plotted in calibration graphs, and they were used to calculate the drug concentration in the unknown sample.

RESULTS AND DISCUSSION

The major difficulty in the analysis of Med 15 and its metabolites is due to their amphoteric properties, which make it difficult to extract them from aqueous media. Extraction from moderately acidic rat plasma at the carefully selected pH of 3.5 provided a good recovery for all the analytes tested: on average, 80.7, 82.6, 99.3 and 90% for Med 15, Med 5, tolmetin and the I.S., respectively. The interassay reproducibility proved to be 5.1% for Med 15, 7.5% for Med 5, and 3.88% for tolmetin (Table I), and linearity was observed for all analytes in the range 0.05–2 μg/ml plasma; higher concentrations were diluted with phosphate buffer (pH 7.4).

The use of acidified plasma, moreover, completely prevents the hydrolysis of Med 15. Extraction recovery tests were carried out by adding Med 15 and the other analytes to previously acidified plasma samples; in this situation no hydrolysis of Med

TABLE I

RECOVERY OF MED 15, MED 5 AND TOLMETIN FROM RAT PLASMA

Each value is the mean (± S.D.) of five determinations.

Compound	Concentration (ng/ml)	Recovery (%)	Coefficient of variation (C.V.) (%)
Med 15	50	80.2(± 5.0)	6.2
	100	81.0(± 2.7)	3.3
	250	79.0(± 5.6)	7.1
	500	88.0(± 1.7)	1.9
	1000	80.8(± 2.7)	3.3
	2000	75.4(± 7.1)	9.4
Mean		80.7	
S.D.		4.11	
C.V.		5.1%	
Med 5	50	71.2(± 8.1)	11.5
	100	85.0(± 7.3)	8.6
	250	80.0(± 9.0)	11.2
	500	86.0(± 4.9)	5.7
	1000	88.4(± 8.1)	9.2
	2000	85.0(± 6.9)	8.1
Mean		82.6	
S.D.		6.20	
C.V.		7.5%	
Tolmetin	50	99(± 2.0)	2.0
	100	102(± 9.3)	9.1
	250	92(± 3.0)	3.3
	500	99(± 2.4)	2.4
	1000	102(± 5.2)	5.1
	2000	102(± 3.7)	3.6
Mean		99.3	
S.D.		3.88	
C.V.		3.90%	

15 occurred. When Med 15 (0.1–5 μg/ml) was incubated at 37°C in fresh rat plasma without acidification, its disappearance was rapid and complete. Med 5 showed very different behaviour when incubated under these conditions. It is very stable in plasma, with a half-life longer than 5 h. The hydrolysis of Med 15 in plasma is enzyme-mediated, since in aqueous solution it proved to be stable for up to 5 h at both 21 and 37°C. The enzyme instability of esterified NSAIAs has been widely documented in the literature [7–10].

Typical chromatograms from (A) standard solutions (concentrations as in point C), (B) drug-free plasma extract, (C) plasma spiked with analytes examined (0.25 μg/ml) and the I.S. (15 μg/ml) and (D) a plasma sample from a rat treated with Med 15 (82 mg/kg) are shown in Fig. 2. There were no interfering peaks from the drug-free plasma samples at retention times of Med 15, Med 5, tolmetin and the I.S. Selectivity was also tested by injecting various NSAIAs, namely sulindac, naproxen, indomethacin, flufenamic acid, ibuprofen and acetylsalicylic acid. None of these interfered with the peaks of Med 15, its metabolites and the I.S.

Chromatogram D in Fig. 2 shows an unknown peak with a retention time of 3.0 min. According to Sumner *et al.* [11] it was identified by gas chromatography–mass spectrometry as MCPA, the major metabolite of tolmetin (Fig. 3) [12]. MCPA fraction peaks eluted from HPLC system were pooled, evaporated, methylated with CH_2N_2 and identified by gas chromatography–mass spectrometry as 5-(*p*-carboxybenzoyl)-1-methylpyrrole-2-acetic acid dimethyl ester.

The HPLC method provided an opportunity for investigating the kinetic profile of Med 15 in rat plasma. Fig. 4 shows the curve of plasma concentration *versus* time obtained from rats treated orally with Med 15 (82 mg/kg). Plasma analysis showed undetectable parent drug at all times tested, as a results of a marked presystemic

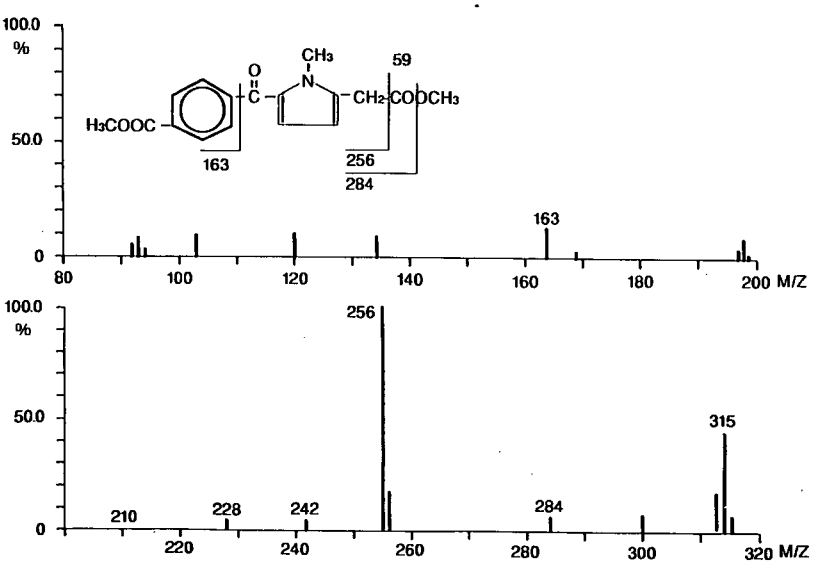

Fig. 3. Mass spectrum of MCPA as its dimethyl ester obtained in electron impact ionization mode (70 eV).

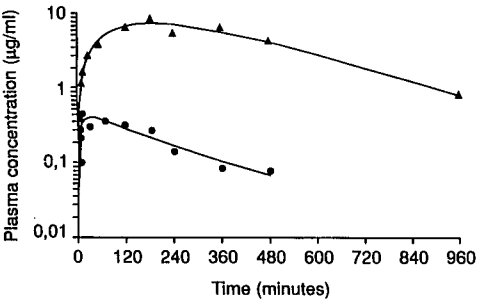

Fig. 4. Plasma concentration–time curves of Med 5 (●) and tolmetin (▲) after oral administration of Med 15 to rats (82 mg/kg), obtained with a computer-assisted process according to the open one-compartment model for oral administration. Each point represents the mean of four findings.

enzyme hydrolysis. A single kinetic curve is suitable for obtaining kinetic results of only qualitative character. The half-life is *ca.* 180 min for both Med 5 and tolmetin and the kinetic behaviour is considered to be monophasic.

CONCLUSION

A sensitive and specific HPLC assay has been developed for the simultaneous analysis of Med 15 and its metabolites. The lowest detectable concentration (0.05 µg/ml) and the good validation allow this method to be used in pharmacokinetic and bioavailability studies in humans.

ACKNOWLEDGEMENTS

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Standardization of cation-exchange clean-up prior to gas chromatography of amino acids

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ABSTRACT

The optimum conditions for the cation-exchange clean-up of amino acids, present in protein hydrolysates, prior to their gas chromatographic determination were investigated. The results of exchaustive study monitoring the amounts of amino acids as N,O(S)-trifluoroacetyl isobutyl esters, revealed that the recovery of amino acids from the column was affected appreciably by either the particle size or the divinylbenzene content of the resins. Quantitative recovery and reproducible determination of amino acids require (i) a 50-fold excess of resin (calculated as equivalent capacity relative to the amino acids present) and (ii) sufficient amounts of eluates: the volumes both of distilled water and of 7 *M* ammonia solution must be about six times the volume of the wet resin applied. Under optimum conditions the recoveries of alanine, glycine, threonine, serine, valine, leucine (isoleucine), proline, hydroxyproline, methionine, aspartic acid, phenylalanine, ornithine, glutamic acid, tyrosine, lysine, arginine and cystine, were quantitative, both without and after hydrolysis, and that of tryptophan was *ca.* 85%.

INTRODUCTION

The advantages of the gas chromatographic (GC) analysis of amino acids in protein hydrolysates are well known [1–11]. Both the capital and running costs of GC equipment are considerably lower, elution times are shorter and the precision and reproducibility are at least the same, or better, in comparison with the classical ion-exchange technique using an automatic amino acid analyser.

From our previous studies [11–14] of the requirements for the GC analysis of protein hydrolysates, obtained from various matrices, we realized that there is a need to reinvestigate some contradictory statements [1,4,7,8] regarding the effect of an ion-exchange "clean-up" step on the recovery of amino acids. The particular aspects of the necessary cation-exchange procedure [8], especially with regard to the recovery of tryptophan, arginine and cystine [4,7], that require consideration are as follows: (i) the ratio of the amount of sample to that of the resin (*i.e.*, amino acid equivalent/resin capacity equivalent), (ii) elution rate, (iii) eluent volume and (iv) resin type, column dimensions and resin bed size.

The aim of this work was to undertake an exhaustive study to optimize the cation-exchange clean-up of protein hydrolysates with respect to the above parameters.

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EXPERIMENTAL

Materials and reagents

All chemicals used were of analytical-reagent grade from Reanal (Budapest, Hungary), Serva (Heidelberg, Germany), Merck (Darmstadt, Germany), Sigma (St. Louis, MO, U.S.A.) and Applied Science Labs. (State College, PA, U.S.A.). Protein matrices were purchased from commercial sources.

Apparatus

A Chromatron (Berlin, Germany) G.C.H.F. 18.3 gas chromatograph equipped with a flame ionization detector and a 2 m × 4 mm I.D. stainless-steel column was used. Nitrogen was the carrier gas at a flow-rate of 60 cm^3/min. The column packing was 3% SE-30 on Chromosorb W (100–120 mesh) (Supelco, Bellefonte, PA, U.S.A.). The column temperature was increased from 105 to 250°C at 6°C/min. The temperatures of the injector and detector were 270 and 270°C, respectively.

Hydrolysis

A 250-μl volume of a stock solution of amino acids (each 500–1000 μg in 250 μl) in 1 *M* hydrochloric acid was evaporated to dryness and 5 cm^3 of distilled hydrochloric acid were added to the residue. The solution of amino acids (10–12 mg in total) was made oxygen free by bubbling nitrogen (99.99%) through for 5 min. The screw-capped bottles were then closed immediately and placed in an oven at 145 $\pm$ 0.5°C for 4 h. Thereafter the hydrolysates were filtered (if necessary) into 25-cm^3 vessels, which could be fitted with either a vacuum distillation device or a reflux condenser, via a ground-glass joint. For filtration glass-fibre paper (grade GF/A; Whatman, Maidstone, U.K.) was used and the insoluble residues were washed with 3 × 1 cm^3 of 0.1 *M* hydrochloric acid. The supernatants were evaporated to dryness for ion-exchange clean-up.

Cation-exchange clean-up

The evaporated residues were dissolved in 1 cm^3 of 0.1 *M* hydrochloric acid and transferred quantitatively with 4 × 1 cm^3 of 0.1 *M* hydrochloric acid onto a cation-exchange resin bed equivalent to $6 \cdot 10^{-3}$ *M* capacity, representing a 50-fold excess of amino acids ($1.2 \cdot 10^{-4}$ *M* in total, calculated with an average molecular weight of 100, *i.e.*, $1.2 \cdot 10^{-2}/100 = 1.20 \cdot 10^{-4}$ *M*). The quality [Amberlite CG-120(H^+), 100–120 mesh; Dowex 50-X8, 30–60 mesh; Varion KS X2, X8, X10, 35–60 mesh] and the particle size (Varion KS X8 35–60, 25–35 and 18–25 mesh) of the resins were varied. In all instances glass columns of 15 cm × 0.75 cm I.D. were used, equipped on the bottom with a glass capillary valve appropriate for the exact adjustment of the flow-rate of the eluent.

The amino acid-containing solution was passed through the resin bed at a rate of 1 cm^3/min, followed 2 min later by 50 cm^3 of distilled water at a rate of 3 cm^3/min.

The amino acids were eluted from the column with 60 cm^3 of 7 *M* ammonia solution at a flow-rate of 1 cm^3/min. The eluate was collected in a 250-ml round-bottomed flask and evaporated to about 1 cm^3. Thereafter the concentrated solution of amino acids was transferred quantitatively with 4 × 1 cm^3 of 0.3 *M* ammonia solution into a 25-cm^3 round-bottomed vessel and evaporated to dryness for derivatization.

Derivatization

To the evaporated residues in the reaction flask, 10 cm^3 of isobutanol [containing 10% (w/w) hydrochloric acid] were added. A reflux condenser was then fitted to the reaction flask and the apparatus was placed in an oil-bath. Esterification took place at 110°C for 60 min. After cooling to room temperature, the solution was evaporated to a syrupy consistency under vacuum in a water-bath at 60°C. To the residue 500 μl of dichloromethane and 1.0 cm^3 of trifluoroacetic anhydride (TFAA) were added and, after sealing the reaction flask acylation was carried out for 10 min at 150°C. Thereafter, the solution of the acylated derivatives was transferred quantitatively into a glass-stoppered, calibrated test-tube (also connected to the vacuum evaporator). The solution of the N,O(S)-TFA esters was evaporated to 500 μl in an ice-bath.

To the residues of the samples dichloromethane and TFAA (in a volume ratio of 4:1) were added, and a stock solution of 0.75 cm^3 was prepared from each. Aliquots of 5–10 μl were injected into the gas chromatograph.

RESULTS AND DISCUSSION

The first step in the model tests was to define the necessary amount and the optimum flow-rate of eluates to obtain maximum (quantitative) amounts of amino acids (Table I).

The types of resin chosen [Table I: Amberlite CG-120(H^+), 100–120 mesh; Dowex 50-X8, 35–60 mesh; Varion KS X8, 35–60 mesh] were based partly on literature data [1–3,6,7] and partly on our preliminary study; all three resins were used after measuring their capacity, ensuring they were present in a 50-fold equivalent excess relative to the *ca.* 12 mg of amino acids present in total. As a result of the measurements the amounts of wetted Amberlite, Dowex and Varion resins were 6, 9 and 9 cm^3, respectively.

The data obtained revealed that the necessary amount of water (fraction I, needed in order to eliminate interfering constituents, such as salts and sugars) contained negligible amounts of amino acids, in particular using the Varion-KS resin (Table I, fraction I). With regard to the essential amount of 7 *M* ammonia solution required, for all three resins it proved to be 60 cm^3 (Table I, fractions II and III). The amounts of amino acids found in the third 3-cm^3 7 *M* ammonia solution (fraction IV) using a flow-rate of 1 cm^3/min were less than the experimental error of the measurements (Table II, relative standard deviations).

The effect of the particle size of the resins on the recovery of amino acids was investigated with Varion KS X8 resins of particle sizes of 35–60, 25–35 and 18–25 mesh (Table II, values in columns A, B and C). As can be seen, no differences as a function of resin particle size were observed.

Regarding the influence of divinylbenzene (DVB) percentages, *i.e.*, the amount of cross-linking in the resin, indicated by X2 (2% DVB), X8 (8% DVB) and X10 (10% DVB) in the resin name, the results obtained were as follows. With the X2 and X8 resins no uptake was observed, except for tryptophan (Table II, columns D and E). The recoveries of all the amino acids tested were quantitative, within the experimental error of the measurements (including cation exchange, derivatization and GC steps). The loss of tryptophan was on average *ca.* 15%.

TABLE I

DISTRIBUTION OF AMINO ACID CONTENT OF MODEL SOLUTIONS MEASURED AS N,O(S)-TFAIBE (DERIVATIVES) IN CONSECUTIVE ELUATE FRACTIONS (I–IV) OBTAINED FROM DIFFERENT CATION-EXCHANGE RESINS

Fractions: (I) 50 cm^3 water; (II–IV) 3 × 30 cm^3 of 7 *M* ammonia solution. Values are means obtained from at least three parallel ion-exchange tests: the differences were smaller than 3.0% [relative standard deviation (R.S.D.)]. Blank values, less than 0.01%. Values in parentheses obtained with an elution rate of 7 *M* ammonia solution of 3 cm^3/min.

Amino acid	Amino acid [% (w/w), expressed as a percentage of the amount applied]											
	Amberlite CG-120(H^+) (100–200 mesh)				Dowex 50-X8 (35–60 mesh)				Varion KS X8 (35–60 mesh)			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
Alanine	–	97.6	2.4	–	0.02	99.3	0.60	0.10	–	99.7	0.20	0.06
Glycine	–	98.2	1.8	–	–	99.6	0.30	0.05	–	99.9	0.07	–
Threonine	0.03	97.0	3.0	–	0.05	98.9	0.80	0.20	–	99.9	0.13	–
Serine	0.06	97.1	2.8	0.08	0.03	99.2	0.60	0.20	–	99.9	0.12	–
Valine	0.02	96.7	3.2	0.06	0.01	98.8	0.90	0.30	–	99.8	0.16	–
Leucine + isoleucine	0.05	96.5	3.2	0.20	0.02	98.0	1.6	0.40	0.01	99.7	0.20	0.08 (1.5)
Proline	0.20	97.4	2.3	0.07	0.10	98.4	1.1	0.40	–	99.9	0.12	–
Hydroxyproline	0.10	97.1	2.7	0.13	0.03	98.5	1.0	0.50	–	99.9	0.13	– (2.0)
Methionine	–	96.8	3.2	–	0.02	97.8	2.0	0.20	0.01	99.7	0.30	–
Aspartic acid	0.08	96.8	3.0	0.11	0.10	97.0	2.5	0.40	–	99.5	0.43	0.06 (0.9)
Phenylalanine	0.14	97.3	2.5	0.08	0.14	96.2	3.1	0.60	0.01	97.8	2.0	0.2 (1.5)
Ornithine	–	95.4	4.6	–	–	100.0	–	–	–	100.0	–	–
Glutamic acid	0.10	95.8	4.0	0.09	0.10	96.2	3.2	0.50	0.02	99.1	0.70	0.19 (0.8)
Tyrosine	0.08	96.9	2.9	0.08	0.07	98.1	1.2	0.60	–	98.5	1.3	0.20
Lysine	0.05	96.7	3.2	–	–	99.5	0.50	–	–	99.9	0.06	–
Arginine	–	95.9	4.0	0.11	–	98.8	1.1	0.05	–	94.5	5.0	0.45 (1.6)
Tryptophan[a]	–	95.3	3.7	1.0	0.09	96.8	3.0	0.10	–	87.7	11.8	0.50 (2.0)
Cystine	–	90.7	9.0	0.30	–	99.2	0.70	0.05	–	96.0	3.5	0.50

[a] Totals, relative to the value without ion exchange, from Amberlite, Dowex and Varion resins, were 75, 85 and 85%, respectively.

With the 10% DVB-containing resin (Table II, column E) considerable shortages, but much lower than reported previously [1,2,4,6,7], were found with respect to the recovery of tryptophan, arginine and cystine (Table II, column E, recoveries in quotation marks).

Comparing the recovery data after hydrolysis (Table II, values A after hydrolysis) with those obtained without hydrolysis, it is obvious that with the exception of tryptophan no additional losses need to be taken into consideration. The recovery of tryptophan after hydrolysis performed in the presence of 3-(2-aminoethyl)indole, followed by cation-exchange clean-up, is currently being studied.

Exhaustive studies have been made to verify the earlier literature data [5,7] concerning the impact of ammonia on the amount of arginine during the evaporation

TABLE II

RECOVERY OF AMINO ACIDS AFTER CATION-EXCHANGE CLEAN-UP APPLYING VARIOUS KINDS OF VARION KS RESINS WITHOUT (A-E) AND AFTER (A) HYDROLYSIS

The main values were obtained from at least three parallel ion-exchange tests, for conditions A–E, before and after hydrolysis, equally. A–E: clean-up test using different kinds of Varion KS resin: A, B and C, Varion KS X8 with particle sizes (A) 35–60 mesh, (B) 25–35 mesh and (C) 18–25 mesh; D and E, Varion KS X2 and Varion KS X10 resin, respectively, with particle size 35–60 mesh. Values in parentheses are percentage recoveries of tryptophan, relative to the value obtained without cation exchange. Values in quotation marks are percentage recoveries of arginine, tryptophan and cystine expressed as percentages of the mean values.

Amino acid	Weighed (μg)	Obtained after elution (μg)								
		Without hydrolysis					After hydrolysis, A	Mean	S.D.	R.S.D. (%)
		A	B	C	D	E				
Alanine	541.9	539	554	546	546	544	550	546	4.4	0.80
Glycine	506.5	506	502	501	506	508	516	508	5.6	1.1
Threonine	511.8	494	516	514	510	523	502	511	9.0	1.7
Serine	515.0	515	515	517	501	511	517	513	5.4	1.1
Valine	539.7	539	539	541	525	535	542	538	5.6	1.0
Leucine + isoleucine	945.9	954	956	953	920	946	946	948	12.9	1.4
Proline	582.4	598	578	588	590	581	593	587	6.6	1.1
Hydroxyproline	534.4	542	549	536	533	539	526	537	6.6	1.2
Methionine	426.7	433	437	428	420	427	426	428	5.0	1.2
Aspartic acid	884.1	880	893	887	877	879	865	883	10.4	1.2
Phenylalanine	819.6	810	828	836	831	810	809	824	12.8	1.6
Ornithine	558.8	569	559	565	559	565	572	564	4.9	0.86
Glutamic acid	771.0	772	762	790	781	770	775	777	9.9	1.3
Tyrosine	527.1	541	528	538	537	532	523	534	5.9	1.1
Lysine	756.0	764	768	801	800	802	746	777	22.0	2.8
Arginine	606.5	597	596	593	590	568[a] "94.8"	604	599	7.9	1.3
Tryptophan	543.7	477 (86.6)	474 (86.0)	477 (86.6)	477 (86.6)	462[a] (83.8) "97.6"	–	476 (85.8)	1.3	0.27
Histidine	791.3	803	798	798	778	760	772	787	15.6	2.0
Cystine	771.2	783	751	756	733	686[a] "90.1"	759	761	17.9	2.3

[a] Left out of the mean.

process: no losses could be found using 1–7 *M* ammonia, in agreement with the present results, with a quantitative recovery of arginine obtained also after the cation-exchange clean-up procedure (Table II, conditions A–D).

CONCLUSION

The surprisingly high losses obtained with Dowex 50-X2 resin [4] for tryptophan (30–35%), cysteine and arginine (30–50%) was decreased for tryptophan (to 30%) and for arginine (to 10%) by applying pressure during elution [7]. The large losses, in general, can be explained by the statement, published in a critical review [8], that "less than half of the publications carried any account of recovery or analytical precision".

The main reason for losses, in our experience, is that the requirement for either a sufficient amount of resin (in the knowledge of its equivalent capacity and the excess of resin needed) or a satisfactory amount of eluate have not been fulfilled.

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CHROMSYMP. 2175

Separation of elastin cross-links as phenylisothiocyanate derivatives

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ABSTRACT

A method has been developed for the separation and quantitation of desmosines in tissue samples. The tissue is treated with cold 10% trichloroacetic acid to remove collagen and hydrolysed in HCl vapours in sealed vials. Preseparation of desmosines from tissue acid hydrolysates is performed on a cellulose column, first eluted with *n*-butanol–acetic acid–water to wash out other amino acids and then with water to recover desmosines. Separated desmosines are then derivatized with phenylisothiocyanate and determined by reversed-phase high-performance liquid chromatography using a gradient system with sodium acetate pH 6.4 and acetonitrile. Desmosines were detected spectrophotometrically at 254 nm. The method was applied to the determination of desmosine in elastin, rat aorta and bovine ligamentum nuchae.

INTRODUCTION

Desmosine and isodesmosine, the elastin-specific cross-linking amino acids, are frequently used as markers of elastin metabolism and its content in tissues. The main problem in their determination is the low proportion of these cross-links relative to other amino acids in elastin (*ca.* two residues per 1000 residues). In elastin-containing tissues their content is even lower, which necessitates tedious enrichment procedures to achieve the necessary accuracy of the assay procedure.

In the first attempts to determine desmosine and isodesmosine, conventional ion-exchange chromatography [1–3], thin-layer chromatography [4,5] and immunoassay procedures [6,7] were used. High-performance liquid chromatography was exploited more recently both with and without precolumn derivatization [8–13]. Of these procedures, derivatization with dansyl chloride [8] or naphthalenedialdehyde cyanide [9] appeared promising, but were used only for the determination of desmosines in pure elastin samples and were not applied to elastin-containing tissues.

Recently Guida *et al.* [14] developed a general strategy for the latter purpose. The hydrolysed elastin or elastin-containing tissue sample was passed through a cellulose mini-column during which the bulk of amino acids were removed. During this operation desmosines were retained and were washed out from the cellulose bed using water as mobile phase. More precisely, after homogenization and extraction of collagen with hot 5% trichloroacetic acid and hydrolysis of the elastin-containing sample with 6 *M* hydrochloric acid, the desmosines were preseparated on a cellulose

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precolumn as mentioned above and were subsequently determined on a Spherisorb ODS-2 column after conversion into dansyl derivatives. The Spherisorb ODS-2 column was eluted with a linear gradient of acetonitrile–25 m*M* phosphate buffer (pH 7.2) from 15 to 60% of the organic modifier. Basically the desmosine enrichment step exploits the procedure described earlier by Skinner [15].

Although in principle the method is applicable to natural elastin-containing samples, there are some problems that require more detailed study. First, although the reported recovery of desmosines reached 87% with a relative standard deviation (R.S.D.) of 2.8% at 50 pmol injected, we have frequently obtained much lower recoveries depending on the batch and provenance of the cellulose sorbent used. Second, determination of the desmosines as dansyl derivatives requires a specialized set-up of the reversed-phase system, which cannot be used directly for conventional amino acid analysis. Therefore, we attempted to combine a commercially optimized amino acid analysis system based on separation of phenylisothiocyanate (PITC) derivatives (PICO-TAG) with an optimized cellulose precolumn enrichment step.

EXPERIMENTAL

Chemicals

Purified desmosines (a mixture of desmosine and isodesmosine) and elastin were obtained from Biotechnology Laboratory, Agricultural Cooperative Enterprises (Letohrad, Czechoslovakia). Standard set of amino acids was purchased from Serva (Heidelberg, Germany). Several batches of cellulose of different provenance were tested in preliminary experiments, and microcrystalline cellulose (Lachema, Brno, Czechoslovakia) was selected for use in further experiments. Acetonitrile (spectrometric grade) was purchased from Janssen (Beerse, Belgium) and *n*-butanol and acetic acid from Merck (Darmstadt, Germany). All other chemicals were obtained from Lachema. Doubly distilled water was used in all operations.

Standards

Stock solutions of desmosines contained 2 μmol/l of the analyte in 0.01 *M* hydrochloric acid. Their precise concentration was determined spectrophotometrically ($\varepsilon = 4900$ at 268 nm for desmosine and $\varepsilon = 7850$ at 278 nm for isodesmosine). Aliquots of desmosines were diluted to 2 ml with 6 *M* hydrochloric acid mixed with a standard amino acid mixture prepared in 0.01 *M* hydrochloric acid (8 ml) and passed through a 4 × 0.5 cm I.D. cellulose minicolumn. This minicolumn was washed first with three 5-ml portions of *n*-butanol–acetic acid–water (4:1:1) and the washings were combined and lyophilized to be used (if necessary) for conventional amino acid determination and for the determination of desmosines in terms of residues per 1000 residues.

Next the cellulose minicolumn was eluted with three 5-ml portions of water, the eluates were combined and lyophilized, the residue was dissolved in 2 ml of 0.01 *M* hydrochloric acid and this solution was used as working standard for the chromatographic calibration.

Analysis of tissue samples

Dried samples of ligamentum nuchae or rat aorta (typically 30–100 mg) were

homogenized in water (1:5, w/v) with a Polytron PT 10-35 homogenizer (Brinkmann, Westbury, NY, U.S.A.). The homogenate was treated with cold 10% trichloroacetic acid and centrifuged at 10 000 *g* (4°C, 10 min). The supernatant was discarded and the residue was treated with 5 ml of 5% trichlororacetic acid at 90°C for 30 min in order to remove collagen (with other tissues this extract can be used for collagen determination by assaying it for its hydroxyproline content). The residue after trichloroacetic acid extraction was washed with acetone in a centrifuge and vacuum dried at 60°C. The dried material was suspended in 2 ml of 6 *M* hydrochloric acid, flushed with nitrogen and hydrolysed in sealed tubes at 110°C for 24 h. The hydrolysate was evaporated to dryness to remove excess of hydrochloric acid, dissolved in 2 ml of 0.01 *M* hydrochloric acid and processed through the cellulose minicolumn as described above.

Elastin samples

A sample of about 2 mg of the protein was dissolved in 2 ml of 6 *M* hydrochloric acid, flushed with nitrogen in a Pyrex tube, sealed and hydrolysed at 110°C for 48 h. The hydrolysed sample was processed as described under *Standards* and *Derivatization and chromatographic equipment.*

Derivatization and chromatographic equipment

Both the desmosines and standard amino acids were separated in the final step as phenylthiocarbamyl derivatives. Lyophilized hydrolysates after the cellulose step were derivatized in a Waters Work Station (Millipore, Milford, MA, U.S.A.) with phenyl isothiocyanate at room temperature for 25 min according to the procedure of Bidlingmeyer *et al.* [16]. Derivatized amino acids were analysed on a PICO-TAG amino acid analysis system (Millipore). Samples were separated by reversed-phase high-performance liquid chromatography using two eluents: eluent A (19.0 g/l sodium acetate trihydrate–0.5 ml/l triethylamine, titrated to pH 6.40 with glacial acetic acid, and subsequently 60 ml of acetonitrile being added to 940 ml of this buffer) and eluent B (400 ml of water added to 600 ml of acetonitrile). The column was conditioned with 100% eluent A for 18 min before every analysis at a flow-rate of 1.5 ml/min. During analysis the flow-rate was 1.0 ml/min. Elution was started with 100% eluent A followed by a linear gradient reaching 46% eluent B at 10 min and 100% eluent B at 10.5 min. Between 10.5 and 12.0 min elution was isocratic with 100% eluent B. The temperature was kept at 38°C and the effluent was monitored at 254 nm.

RESULTS AND DISCUSSION

Cellulose precolumn sample enrichment

As demonstrated in Fig. 1A–C, the bulk of common amino acids are eluted with the organic mobile phase [*n*-butanol–acetic acid–water (4:1:1)]. Even with different batches of cellulose we have never observed leakage of desmosines into the first three organic phase washings. The third organic phase washings of the cellulose precolumn are virtually always devoid or contain very little of any of the common amino acids (less than 2.0 mol% of originally loaded Asp, Glu, Hypro, Ser, Gly, His, Thr, Ala, Pro, Tyr, Val, Phe and Lys and less than 5.0 mol% of originally loaded Arg).

Fig. 1D–F demonstrate typical amino acid profiles obtained with subsequent elution with water. Particularly the first aqueous washings contain considerable

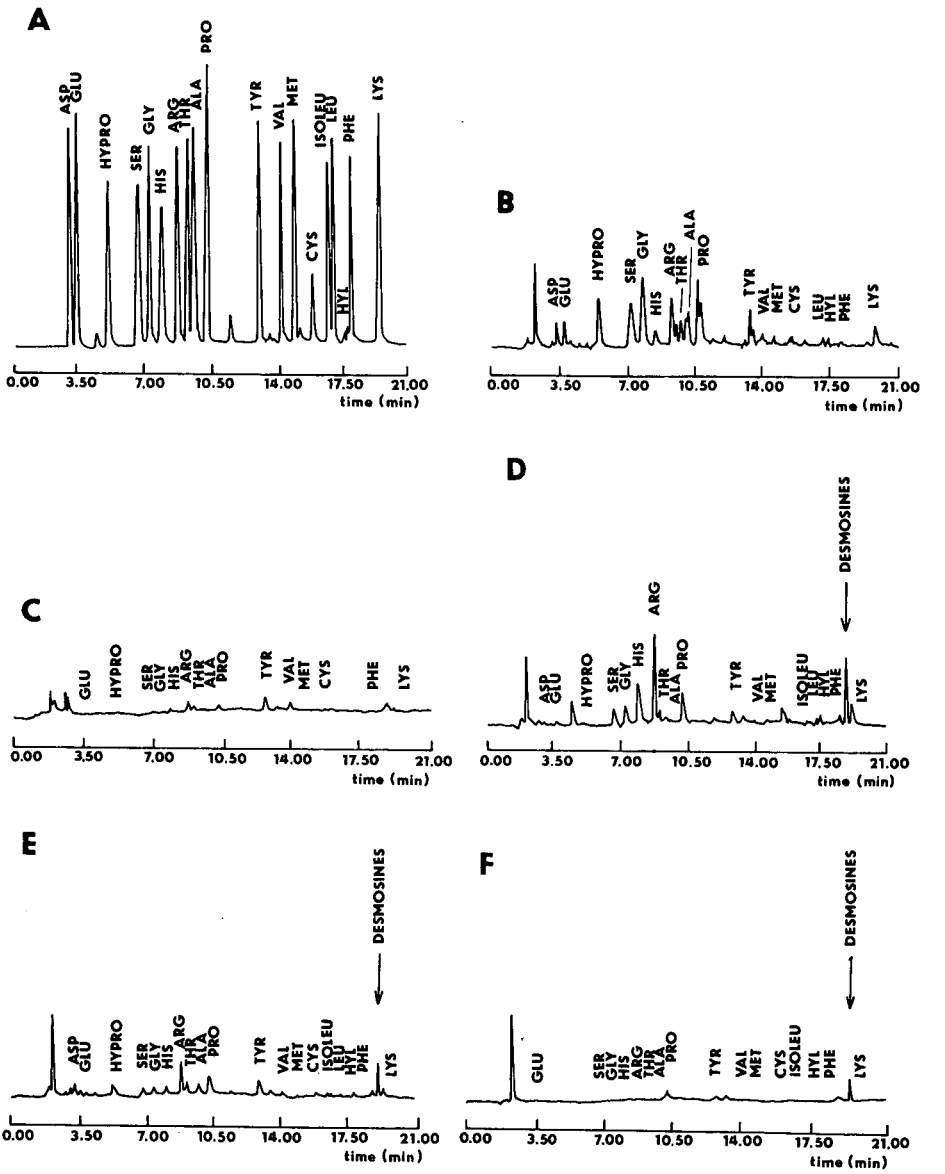

Fig. 1. Cellulose precolumn sample enrichment. A–C: amino acid analysis of the first three fractions eluted with *n*-butanol–acetic acid–water (4:1:1); note that the third organic phase washings are devoid of common amino acids. No washing out of desmosines can be observed. D–F: stepwise elution of desmosines with water, amino acid analysis of the three eluted fractions.

amounts of His (8.24 ± 1.23 mol%), Arg (16.23 ± 2.8 mol%) and Pro (6.32 ± 0.76 mol%) (±S.D., $n = 8$) of the amount loaded (2500 pmol).

The second aqueous washings contain only traces of the originally loaded

amount of contaminating common amino acids and the third wash is completed only to increase further the recovery of desmosines.

As has been mentioned, no desmosines are recovered with the *n*-butanol–acetic acid–water elution. In the first aqueous washings 80.03 ± 2.20 mol% of desmosines were recovered; an additional 6.24 ± 0.31 mol% were recovered in the second and 2.04 ± 0.7 mol% in the third washings (±S.D., 250 pmol injected), thus reaching a total recovery of 88.31 mol%. The within-day and day-to-day reproducibility (R.S.D.) of the method was 2.2 and 5.1%, respectively (with 25 pmol of desmosines injected).

By cellulose precolumn treatment an enrichment factor of $5 \cdot 10^3$ can be easily achieved with a limit of detection of 2.5 pmol of desmosines (signal-to-noise ratio 3 at attenuation × 1).

Calibration graphs for 20–2500 pmol of phenylisothiocyanate derivatives of desmosines exhibited good linearity of plots of peak area *vs.* amount of analyte injected with a correlation coefficient of $r = 0.997$.

With some batches of cellulose (particularly from other sources) the data may vary and it is therefore recommended to use for a series of experiments a single batch of cellulose that has been adequately tested before being put into routine use.

Analysis of elastin containing tissues

Typical examples of the determination of desmosines in elastin hydrolysate, rat aorta and bovine ligamentum nuchae are presented in Fig. 2A–C. With standard addition of desmosines the recovery of these cross-linking amino acids was 88.24 ± 4.7% (±S.D., $n = 20$, ligamentum nuchae), which is similar to the value obtained with an artificial amino acid mixture. Also, the amount of desmosines found increases linearly with the amount of tissue taken for analysis ($n = 20, r = 0.996$). The minimum amount of tissue needed for reliable determination of desmosines in elastin-rich tissues was 30 mg for ligamentum nuchae and 50–100 mg for rat aorta. With separate samples of dried aorta the R.S.D. obtained was 6.3% ($n = 20$). The proportion of contaminating common amino acids present in the combined aqueous eluates differs considerably from the contaminants seen when working with standard amino acid mixtures, which obviously reflects the amino acid composition of the proteinous material analysed.

In comparison with the method published by Guida *et al.* [14], the present procedure has similar analytical parameters; its advantage is the exploitation of commercially available equipment for amino acid analysis. The unsatisfactory results we obtained at the beginning with the above-mentioned procedure are obviously due to the differences in the properties of the cellulose used. With some batches of cellulose we observed considerable retention of common amino acids even after repeated elution of the cellulose precolumn with *n*-butanol–acetic acid–water (4:1:1). Two, or better three, washes (as described in this paper) are necessary to eliminate as much of the common amino acids as possible. No leaking of desmosines into the organic phase washings was observed, thus allowing multiple elution of the cellulose precolumn. Also, repeated elution with water is needed to obtain good recoveries of desmosines.

Even with the high sample enrichment achieved by using the cellulose precolumn, in naturally occurring samples removal of non-collagenous and collagenous proteins by trichloroacetic acid treatment is inevitable as otherwise the content of desmosines even in elastin-rich tissues is for too low to allow direct analysis of the tissue.

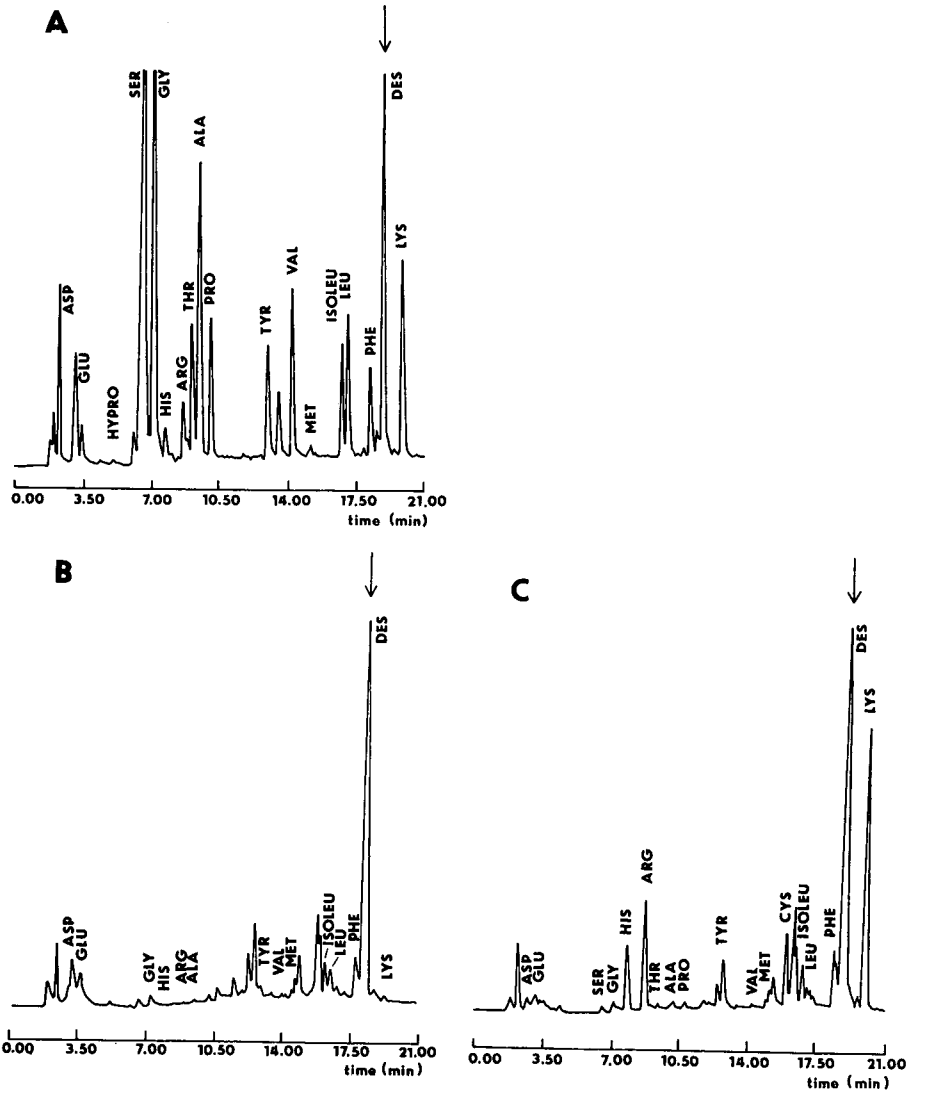

Fig. 2. Typical examples of the determination of desmosines in (A) elastin hydrolysate, (B) ligamentum nuchae and (C) rat aorta.

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Recent advances in liquid chromatography–mass spectrometry and capillary zone electrophoresis–mass spectrometry for protein analysis

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ABSTRACT

The utility of the combination of separations techniques, such as liquid chromatography and capillary zone electrophoresis, with mass spectrometry in applications involving protein analysis is discussed. The use of continuous-flow fast atom bombardment and electrospray ionization mass spectrometry is compared for the analysis of tryptic digests. For liquid chromatography, both microbore and slurry-packed capillary bore columns were used to separate peptides from proteolytic digests.

INTRODUCTION

Mass spectrometry is playing an ever widening role in the investigation of analytical and structural problems encountered in the biological sciences. To date, a large number of separations techniques have been combined with mass spectrometry (MS) to provide the biotechnologist with very sensitive and specific tools with which to unravel complex biological processes. These include gas chromatography, high-performance liquid chromatography (HPLC), electrophoresis, thin layer chromatography, supercritical fluid chromatography, counter current distribution, gel permeation chromatography, and microdialysis. Over the past six years, instrumental combinations such as liquid chromatography–mass spectrometry (LC–MS) and capillary zone electrophoresis–mass spectrometry (CZE–MS) have made substantial gains both in their capabilities and mechanical utility and are becoming important tools for the separation and detection of molecules in complex mixtures.

The combination of liquid chromatography with mass spectrometry requires the use of an interface to handle the high gas loads which are produced by vaporization of the LC solvent. For example, 1 ml of liquid water gives approximately 1200 ml of gas [standard temperature and pressure (STP)]. However, most commercial MS systems are only able to handle a small fraction of this with standard pumps. A number of potential solutions to this high gas load problem have been devised; (i) evaporation of the solvent outside of the mass spectrometer, *i.e.*, use of a moving belt

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or similar transport system, (ii) direct introduction of a fraction of the eluent such as that used in the direct liquid introduction (DLI) technique, (iii) use of micro columns to provide lower flow-rates as utilized in continuous-flow fast atom bombardment (CF-FAB), (iv) use of additional pumps and heat, as in thermospray, and (v) use of an atmospheric pressure ionization technique in which a portion of a spray of ions is sampled by the spectrometer, as in electrospray. Generally, all of these techniques have been utilized by at least one MS interface, albeit with variable success.

One of the most useful applications for LC–MS analysis in biotechnology is that of peptide mapping. In this procedure, a protein or polypeptide is digested with a proteolytic enzyme such as trypsin, chymotrypsin, subtilisin, or another similar protease. An aliquot of the digest is then injected directly into the liquid chromatograph, usually without further sample concentration or clean-up. Separations are generally accomplished using gradient elution systems and reversed-phase columns, often C_8 and C_{18} columns, eluted with water and an organic phase such as acetonitrile. The mass spectrometer is scanned continuously, usually between m/z 200 and 2000. Data analysis consists of the identification of all of the specific masses, usually protonated molecular species. The focus of this paper will be on the use of LC–MS, and to some extent CZE–MS, for the analysis of peptides and peptide digests. Data obtained from analyses by LC–MS will be compared to that obtained for the direct analysis of the hydrolysis mixture, *i.e.*, direct injection of the sample without the use of an LC column. A comparison will also be made of LC–MS data obtained from electrospray and CF-FAB.

EXPERIMENTAL

Liquid chromatography was performed on an Applied Biosystems Model 130A microbore liquid chromatograph, modified for LC–MS and operated as previously described [1]. Microbore separations were done using a Brownlee Aquapore RP-300 (C_8) column, 50 × 1 mm I.D.), at gradient flow-rates of approximately 25 μl/min. Typically, gradient elutions were performed from 0–20% solvent B in 10 min, 20–45% B in 35 min, and 45–100% B in 10 min, holding 100% B for 2 min. For CF-FAB solvent A was water–glycerol–trifluoroacetic acid (TFA) (95:5:0.1); solvent B was acetonitrile–water–glycerol–TFA (60:35:5:0.1). For electrospray the glycerol in the eluents was replaced by water. The UV detector was set to monitor at 215 nm. The eluent from the column was split 4:1, allowing a flow of 5 μl/min to enter the source of the mass spectrometer. Sample injections containing 300 pmol were applied to the column, and 60 pmol were actually transferred into the ion source.

Microcapillary LC separations were obtained using a 320 × 0.25 mm I.D. fused-silica capillary slurry packed with Spherisorb ODS2 (3 μm). The outlet of the microcapillary column was fitted with a 0.025 mm I.D. capillary about 25 cm long. This outlet capillary was in turn connected via a short sleeve of PTFE tubing to a 1 m length of 0.048 mm I.D. fused-silica capillary (Polymicro Technologies, Tucson, AZ, U.S.A., not deactivated) that served as the transfer line to the mass spectrometers. About 5 cm from the beginning of this transfer line a small area from which the polyimide coating had been removed served as the window for on-line UV detection at 210 nm (Applied Biosystems). A Waters gradient HPLC system consisting of Models 590 and 510 pumps and a Model 680 solvent programmer was used to pro-

duce gradients at a flow-rate of 1.2 ml/min. The solvent was then split prior to the injector (Rheodyne 8125, variable volume). Two Waters Nova-pak C_{18} (15 × 0.39 cm I.D.) columns in series were attached to the high flow side of the split tee, thus providing the back pressure needed to achieve a flow-rate of 2–2.5 μl/min through the microcapillary column. The following gradient program was used: 0–50% B over 7.5 min, 50–88% B over 22.5 min, then stepped and held at 100% B. For LC–MS with CF-FAB, solvent A was water–glycerol–TFA (95:5:0.1) and solvent B was water–glycerol–acetonitrile–2-propanol–TFA (39:5:45:11:0.1). The glycerol was replaced by water for LC–MS with electrospray. A 0.1-μl injection volume equivalent to 50 pmol of digest was used to load the microcapillary column. No post-column split was necessary.

LC–CF-FAB-MS conditions were used as previously reported [1] and were performed on a Finnigan MAT 90 high-performance magnetic mass spectrometer. For LC–MS analyses, the spectrometer was scanned in one of three mass ranges for each sample injection; *e.g.* m/z 350–1100, 1100–1700, and 1700–2650 at a scan speed of 10 s/decade and at a resolution of approximately 1200.

Electrospray ionization was performed on a Finnigan MAT TSQ 70 quadrupole mass spectrometer. For LC–MS analysis, the general operating conditions previously reported [2] were employed except that a sheath liquid, isopropyl alcohol, was used at a flow-rate of 2 μl/min. The instrument was scanned from 300 to 1500 a.m.u. in 5 s. The flow-rates for the columns were 5 μl/min for the microbore column and 2.1 μl/min for the packed capillary column. For direct injection of a proteolytic digest, approximately 50 pmol of digest in 1 μl of 0.1% TFA was injected into a solvent carrier consisting of water–acetonitrile–TFA (80:20:0.1). The flow-rate was 5 μl/min, with an additional sheath liquid (isopropanol) flow of 4 μl/min.

The CZE–CF-FAB-MS apparatus was essentially the same as that reported previously [3]. The CZE capillary (Polymicro Technologies) was 90 cm × 50 μm I.D. For the analysis of tryptic digests, approximately 25 pmol of the digested protein mixture was pneumatically loaded into the capillary. Electrophoresis was performed at 15 kV (8 μA) with 40 m*M* sodium citrate and 40 m*M* sodium chloride, pH 2.5, as the buffer. The CF-FAB carrier solution, present in the cathodic reservoir interface was composed of water–glycerol–acetonitrile (92:5:3), containing 2.3 m*M* acetic acid and 1 m*M* ammonium hydroxide. This solution was allowed to flow into the ion source at 5 μl/min.

RESULTS AND DISCUSSION

LC–MS

For LC–MS, generally three column options are available; full bore, microbore, and capillary bore. One of the major differences among these columns is the solvent flow-rates that are used. As a result of the high gas load that the evaporated solvent places on the vacuum system, microbore and capillary bore columns are generally favored for on-line mass spectrometry applications. While full bore columns can be used, ionization techniques such as FAB and electrospray would require solvent flows to be split 1/50 to 1/100, thereby significantly affecting sensitivity. On the other hand, only split ratios of 1:3 to 1:4 need be used with microbore columns and no splitter at all for capillary bore columns.

We have found that use of a microbore column at flow-rates of approximately 25 μl/min provides a satisfactory balance between chromatographic performance and a low sample-split ratio. The steepness or rapidity of the gradient program is an extremely important parameter which the investigator can utilize to achieve specific

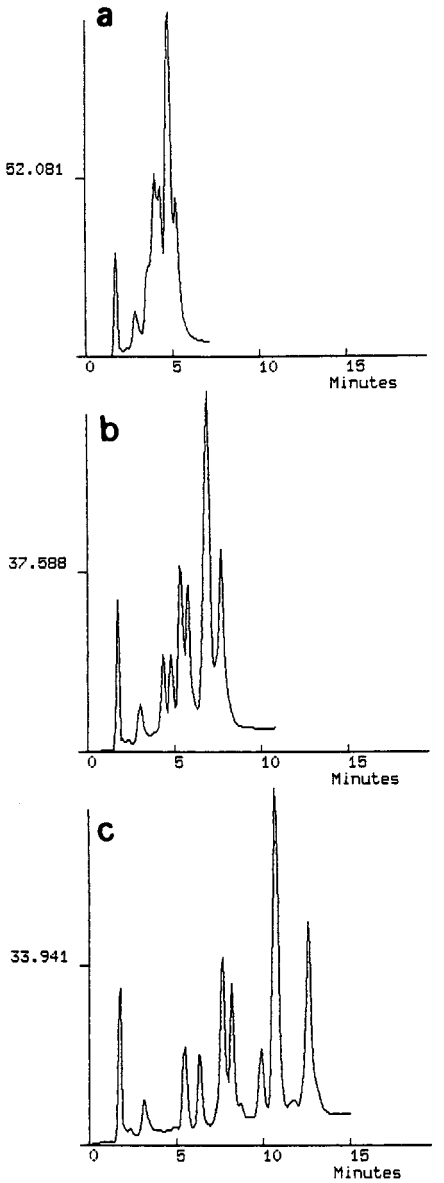

Fig. 1. The gradient elution of a sample of the tryptic digest of horse heart cytochrome *c* using UV (210 nm) detection on a 1 × 50 mm Aquapore RP-300 (C_8) column. The following gradient programs were used: (a) 5–40% B, 2.5 min; 40–60% B, 1.0 min; 60% B, hold; (b) 5–40% B, 5.0 min; 40–60% B, 1.0 min; 60% B, hold; (c) 5–40% B, 10.0 min; 40–60% B, 1.0 min; 60% B, hold. Approximately 100 pmol of peptides were injected into the column. Chromatographic conditions are given in the experimental section.

types of analyses. Fig. 1 shows the separation of a cytochrome *c* tryptic digest on a 50 × 1 mm I.D. C_8 column using three different gradient programs. The highest resolution separation is, of course, achieved with the shallower gradient, and would be preferred if component separation and collection are required. On the other hand, the most rapid gradient program is effective if the analyst wishes to simply separate the compounds in the digest from salt or large molecules, as might be required in on-line process monitoring where many aliquots need to be analyzed in a narrow time domain. In fact, most of the mass spectral data obtained from the slow, higher resolution LC analysis is obtainable from the rapid gradient analysis as well. However, ion suppression problems, coelution of compounds of the same mass, and lack of the ability to collect fractions of separated compounds remain drawbacks of the rapid elution technique.

CF-FAB–MS

CF-FAB is a technique that is based on the flow of a carrier solvent into the source of a mass spectrometer with subsequent atom bombardment of the liquid as it flows over the surface of a target [4,5]. Liquid samples may be superimposed on this flow by flow-injection techniques, or this carrier flow can be the effluent of an LC. A typical microbore LC–CF-FAB set-up is shown in Fig. 2. A splitter is used prior to the sample injection valve so that gradients can be formed rapidly and mixed efficiently before being put through the column. Typically, gradients are formed at 200–500 μl/min and then split so that approximately 25 μl/min flow through the injector to the column. After the column, a 1:4 split of the eluent is used to give 5 μl/min flow into the MS ion source. The remaining 20 μl/min is allowed to flow through a UV detector and on into a fraction collector, if desired. For the separation of peptides, a TFA–acetonitrile gradient system is often used with a C_8 column. Both eluting solvents contain 5% (v/v) glycerol to help stabilize the flow of liquid over the FAB target surface.

For the purpose of comparing the performances of microbore and capillary columns in LC–MS applications, and CF-FAB with electrospray, we have chosen to use the tryptic digest of human growth hormone (hGH) as a typical example. A detailed analysis of the tryptic digest of this protein has been published by other

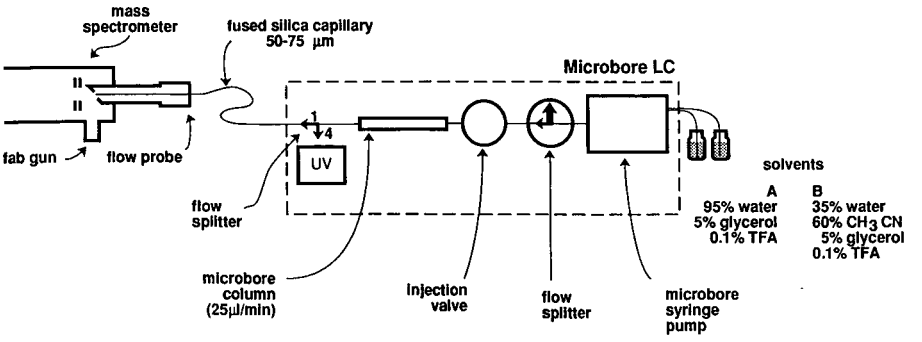

Fig. 2. LC–CF-FAB-MS instrumental arrangement for microbore analyses. (Reprinted with permission from ref. 1.)

investigators [6]. Twenty-one tryptic peptides are normally present in the limit digest, although several others can be seen at low levels presumably due to the action of contaminating proteases. In some of the figures that include chromatograms, we have selected only six of these fragments in order to simplify the comparisons. The $(M+H)^+$ values of these are *m/z* 383, 693, 1254, 1362, 1400, and 2055. These particular peptides not only represent a distribution of different molecular weights, but also have retention times which fall throughout the entire time period of peptide elution.

Chromatograms for the microbore LC–CF-FAB-MS analysis of the tryptic digest mixture of hGH are shown in Fig. 3. The mobile phase and gradient conditions are described in the experimental section. The amount of sample injected onto the microbore column was 300 pmol, with 60 pmol flowing into the mass spectrometer. The top panel of the figure shows the UV absorbance trace at 215 nm, and the bottom panel the selected ion chromatograms for the six peptides listed above. The ion chromatogram plots are generated by superimposing the six selected ion chromatograms on a single axis. Thus, each selected ion chromatogram is independently normalized and in such a summary plot, absolute intensities are not given. We prefer such a plot

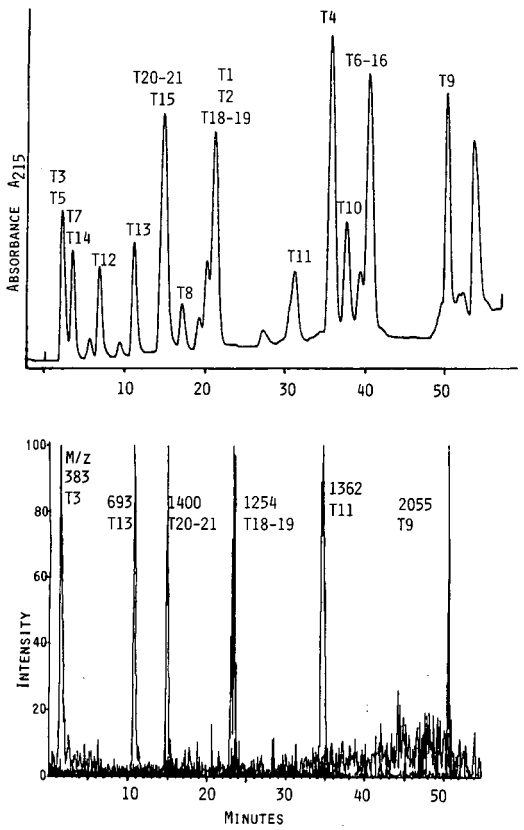

Fig. 3. The microbore LC–CF-FAB-MS analysis of 60 pmol of hGH tryptic digest; (top) the UV absorbance profile, and (bottom) the selected ion chromatograms, independently normalized, for six of the peptides in the mixture. Chromatographic conditions are given in the experimental section.

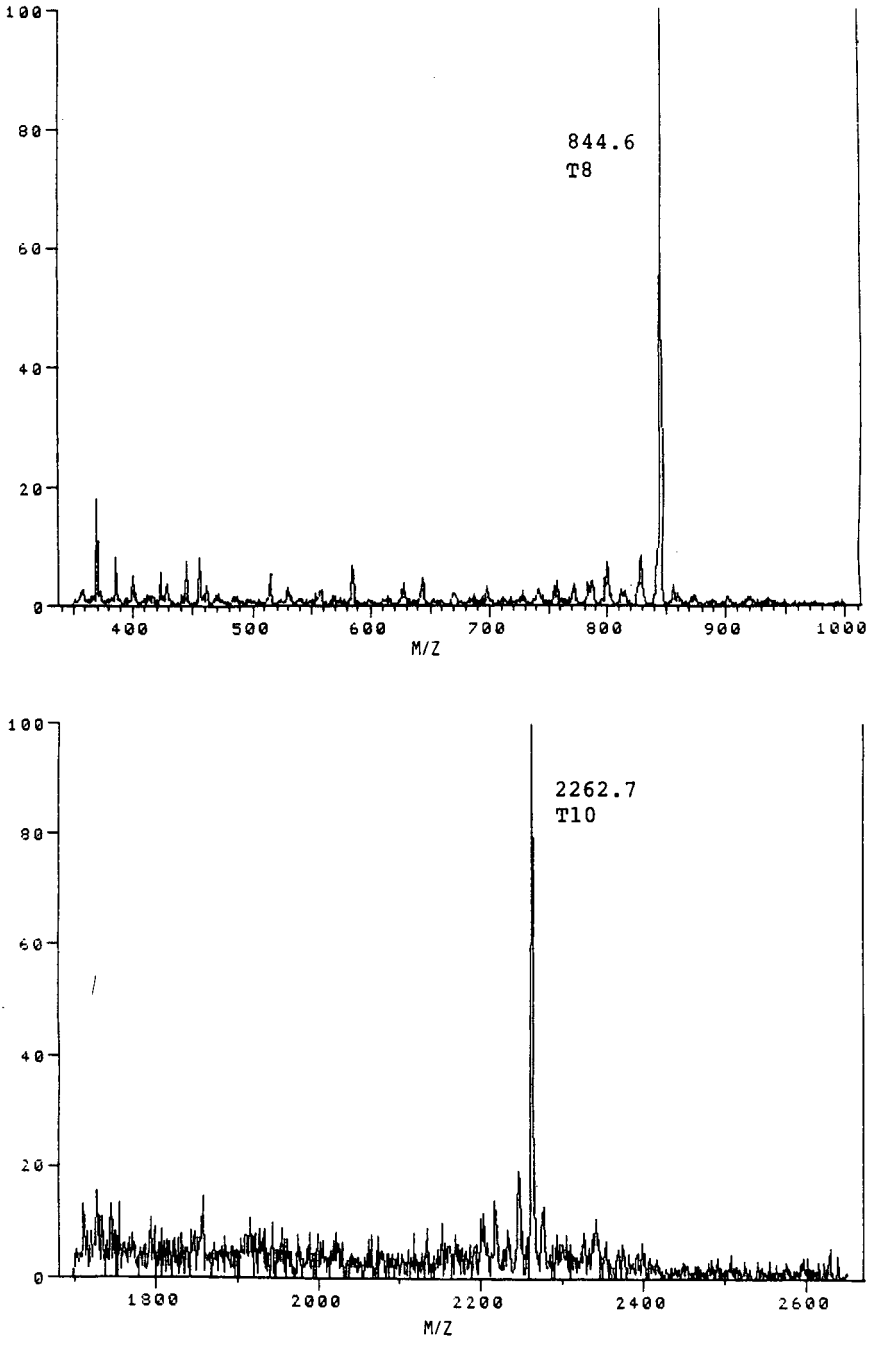

Fig. 4. The CF-FAB mass spectra of two peptides from the digest shown in Fig. 3; *m/z* 844, fragment T8, and *m/z* 2262, T10. Approximately 60 pmol of peptides were transmitted into the mass spectrometer.

because it quickly and easily indicates the position of a given mass, the essential parameter in the data workup. Of course, absolute ion intensities can be obtained from the individual selected ion chromatograms used to make this summary plot.

The analysis shown in Fig. 3 was obtained from a high-performance magnetic double-focusing mass spectrometer. In order to achieve the optimum signal-to-noise ratio in recording these data, the instrument was scanned in three different scan ranges; 350–1000, 1100–1500, 1700–2650. Each of these mass ranges was recorded using a separate LC–MS analysis and, therefore, the ion chromatogram shown in Fig. 3 represents the sum of these three sample analyses. The UV trace in Fig. 3 is from one of these analyses. Four of the individual tryptic fragments are found in disulfide linkage; T6–T16 and T20–T21. The mass spectra of two peptides identified in the analysis are presented in Fig. 4 to illustrate the signal-to-noise ratio obtained in the mass spectrometric analysis of 60 pmol of the digest. The ion at *m/z* 844 is the peptide T8 (tryptic fragment 8) and *m/z* 2262, the peptide T10. The separation and analysis of 50 pmol of the hGH tryptic digest injected onto a packed capillary column (C_{18}, 32 cm × 0.25 mm I.D.) gave the same results as that obtained from the microbore LC–MS analysis, *i.e.*, all of the expected peptides were identified and were eluted over a time of about 50 min (data not shown).

The advantages of CF-FAB are several; it produces relatively intense $(M + H)^+$ ion species for the various peptides, providing suitable ion intensities for MS–MS analyses at the 5–50 pmol level, it is compatible with many types of buffers normally used in biological reactions, and it can be used with either aqueous or organic solvents. The CF-FAB set-up is generally available as a relatively inexpensive option and can be retrofitted to most modern MS systems.

Electrospray MS

Electrospray ionization is an MS technique which produces ions in a spray of microfine solvent droplets at atmospheric pressure and in a high electric field [7,8]. In the source used in this study, ions created in the spray are transported through a glass capillary into the ion source of a mass spectrometer, as shown in Fig. 5. This partic-

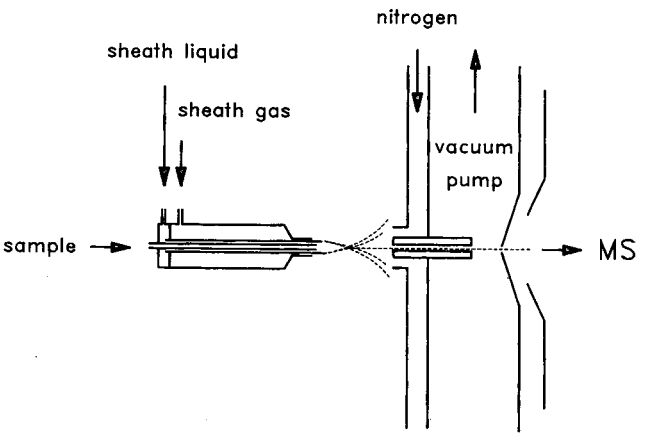

Fig. 5. The schematic diagram of an electrospray ion source. The microfine droplet spray containing ions is shown as dotted lines.

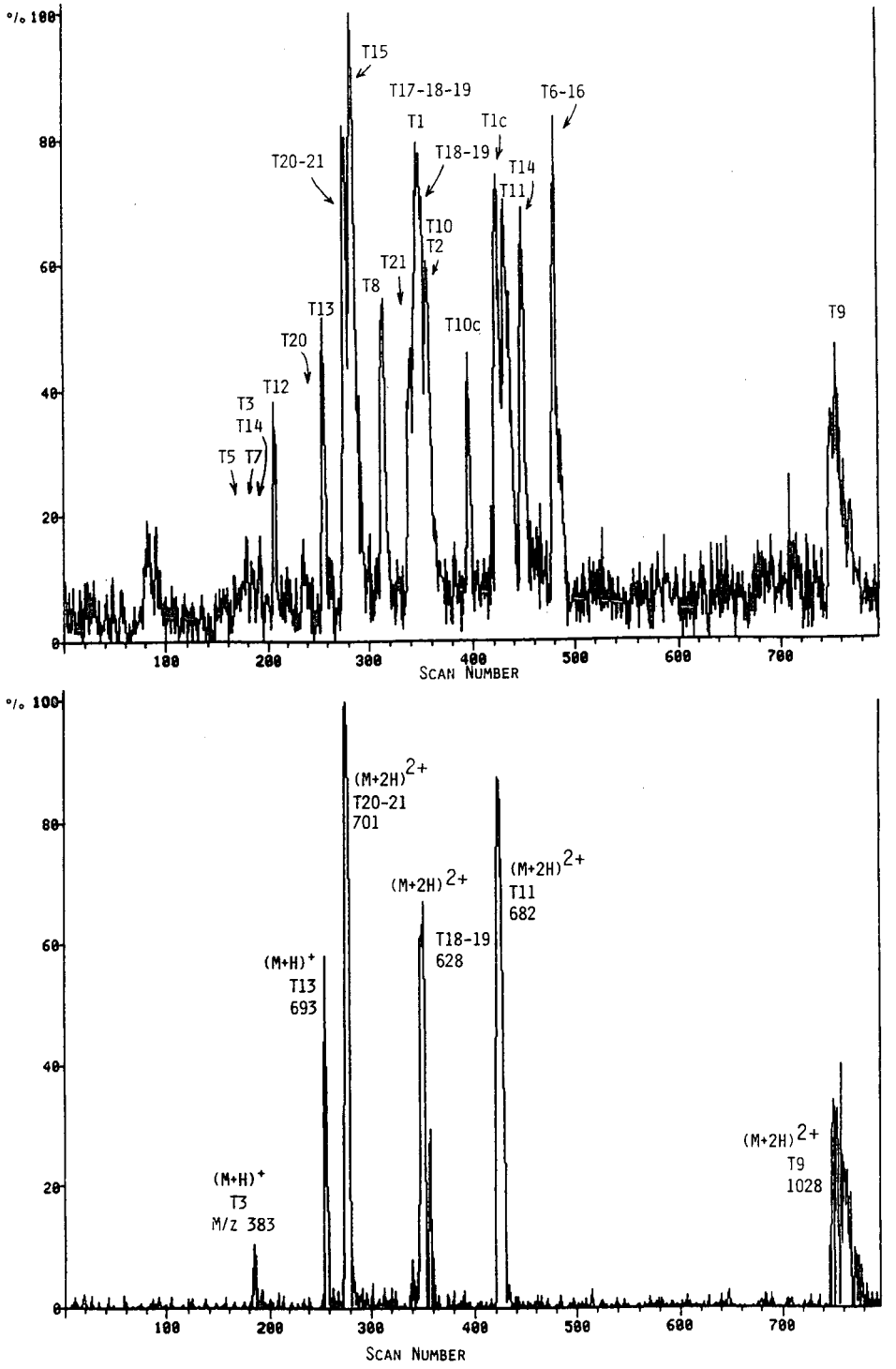

Fig. 6. The LC-electrospray analysis of 50 pmol of the tryptic digest of hGH using the capillary bore C_{18} column. (Top) the total ion chromatogram with background subtracted, and (bottom) the selected ion chromatograms of six peptides in the mixture showing their relative intensities. Chromatographic conditions given in the experimental section.

ular source contains three concentric capillary tubes; the inner capillary delivers the sample or LC eluent, the middle capillary delivers a sheath liquid, such as isopropyl alcohol, to stabilize the spray formation under LC conditions, and the outer capillary delivers nitrogen gas to help form and define the spray so it may be efficiently sampled by the mass spectrometer. Generally, quadrupole mass spectrometers are used for this ionization method because they have relatively low voltage ion sources. Like other MS ionization methods which directly take liquid from the LC, electrospray also works best at relatively low flow rates, *i.e.*, about 2–5 μl/min.

Ion chromatograms obtained from the separation and analysis of 50 pmol of the hGH tryptic digest injected onto a C_{18} slurry packed capillary column are shown in Fig. 6. The upper panel gives the total ion chromatogram for the peptides in the mixture, and the lower panel, the selected ion chromatograms for the six peptides showing their relative intensities. Four of the peptides in the selected ion chromatogram were detected as $(M+2H)^{2+}$ ions, *i.e.*, peptides of molecular weight 1253 (T18, 19), 1361 (T11), 1400 (T20–T21), and 2054 (T9) were recorded as *m/z* 628, 682, 701, and 1028, respectively. Fig. 7 shows the selected ion chromatograms for the singly and doubly charged molecular species of tryptic fragment T13. The peak at *m/z* 347 is the $(M+2H)^{2+}$ species and that at *m/z* 693, $(M+H)^{+}$. The mass spectrum taken from this chromatographic peak is shown in Fig. 8, upper panel. The lower panel of this figure shows a second mass spectrum for two tryptic fragments linked by

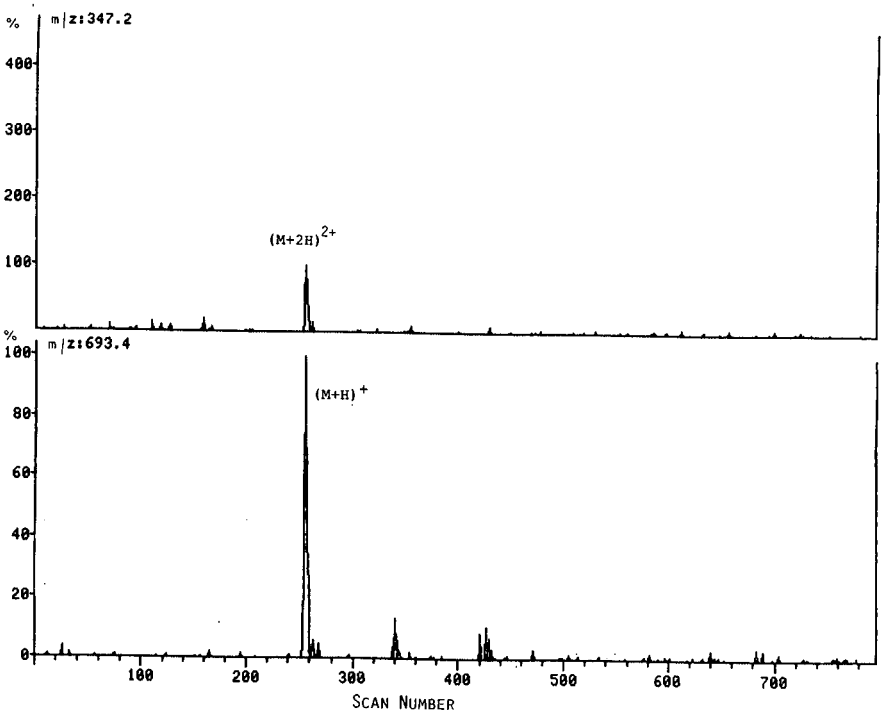

Fig. 7. The selected ion chromatograms for peptide fragment T13 from the analysis given in Fig. 6 showing both the singly (*m/z* 693.4) and doubly charged (*m/z* 347.2) ion chromatograms.

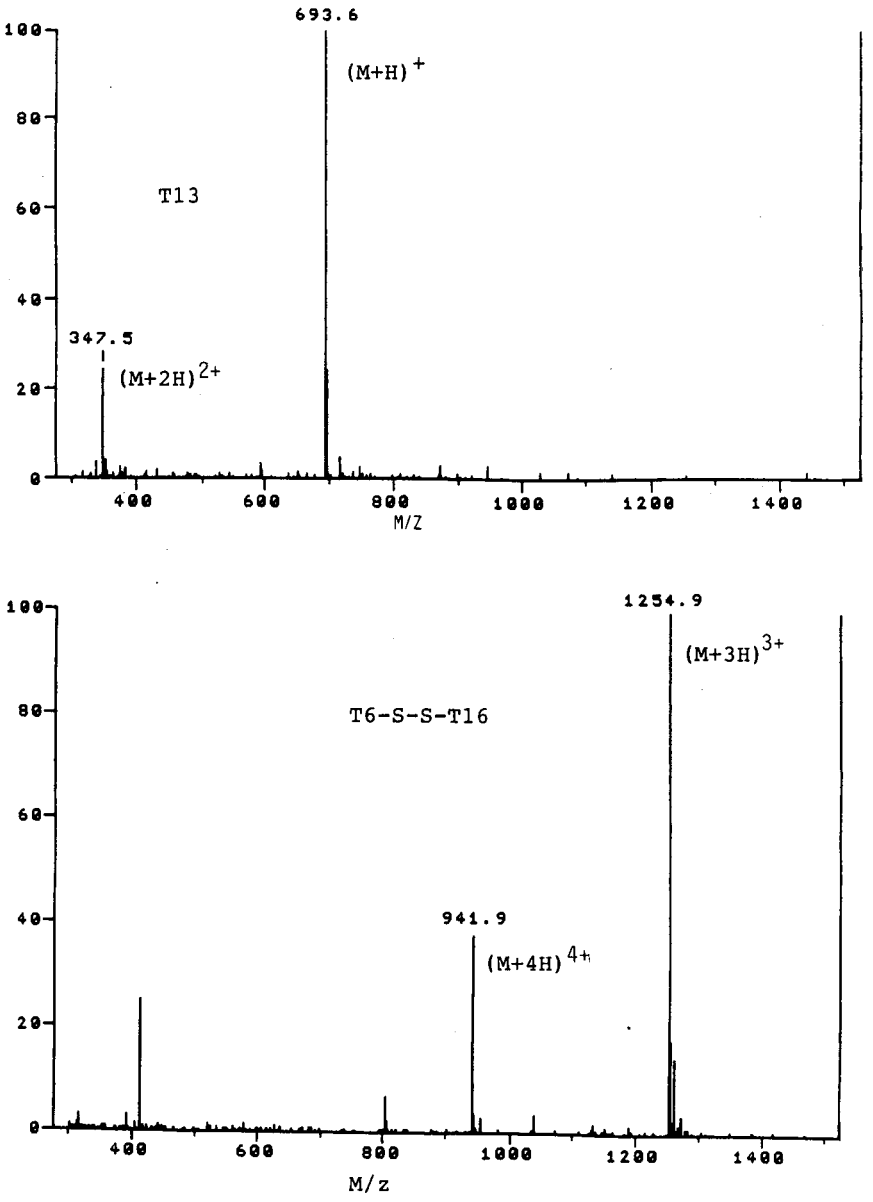

Fig. 8. The electrospray mass spectra for two peptide fragments from the LC–MS analysis shown in Fig. 6.

a disulfide bridge (T6–S–S–T16) at m/z 1255 for $(M+3H)^{3+}$ and m/z 941 for $(M+4H)^{4+}$.

One of the common analysis procedures for a tryptic digest is the direct analysis of an aliquot either by FAB or, more recently, electrospray without the use of an LC column. This procedure is generally termed FAB-mapping when the FAB ionization method is used. Although it takes considerably less time and effort to perform such an

analysis, the results are inferior in comparison to the information obtained from the LC–MS system. This is shown in Table I for the comparison of the capillary LC–electrospray MS analysis of 50 pmol of the hGH digest compared to the direct injection of 50 pmol/μl of the digest into the electrospray source. It is seen that the LC–MS analysis is superior in permitting the identification of 20 of the 21 peptides in the scan range and at higher comparative intensities than that obtained by direct injection, where only 11 of the 21 peptides were identified. The LC system thus performs an extremely important function for the mass spectrometric ionization process in that it separates compounds entering the ion source in time, minimizing ion suppression effects that often occur when many compounds arrive in the source at the same time.

As indicated above, one of the great advantages of electrospray over other ionization techniques is its ability to form multiply charged ions. Since mass spectrometers measure mass-to-charge (m/z) ratios, large molecules whose singly charged molecular species normally fall outside the effective mass range of the spectrometer can be recorded as multiply charged ions. For example, a sample of soybean trypsin inhibitor (molecular weight 20 091) will form a distribution of multiply charged ions

TABLE I

ANALYSIS OF hGH TRYPTIC DIGEST BY ELECTROSPRAY MASS SPECTROMETRY, COMPARING LC–MS AND DIRECT INJECTION ANALYSIS

Tryptic fragment	Molecular ion observed	Relative ion intensity[a]	
		LC–MS[b] analysis of digest	Direct injection of digest[c]
4	2343^{2+}	+ + +	+
9	2056^{2+}	+ + +	+ +
11	1363^{2+}	+ + +	+ + +
14	627^{2+}	+ + +	+ + +
15	1491^{2+}	+ + +	+ + +
18(19)	1255^{2+}	+ + +	+ + +
21	786^{2+}	+ + +	–
1	931^{1+}	+ +	+
6	2618^{3+}	+ +	–
8	845^{1+}	+ +	+
10	2264^{3+}	+ +	–
20–S–S–21	1401^{2+}	+ +	+
2	980^{1+}	+	+
3	383^{1+}	+	–
6–S–S–16	3765^{4+}	+	–
12	773^{1+}	+	–
13	693^{1+}	+	+
20	618^{1+}	+	–
5	404^{1+}	+	–
7	762^{1+}	+	–
16	1150^{2+}	–	–

[a] Relative ion intensity shown as highest (+ + +) to lowest (+). Ions not observed noted by (–).

[b] Data from capillary LC–MS analysis shown in Fig. 6; 50 pmol of peptides transmitted into the MS. Sample solution contained 50 pmol in a 1 μl injection.

containing charged molecules from 16^+ to 22^+ [9]. These multiply charged species are, of course, related to a single molecule species and this multiply charged spectrum may be deconvoluted to give the molecular weight of the protein. Because these multiply charged species mainly fall between m/z 800 and 2000, relatively low cost quadrupole instruments can be used quite effectively for high-molecular-weight measurements of 10 000–50 000 daltons or more.

CZE–MS

CZE–MS is of great interest and potential in the field of analytical biochemistry because of its relatively high separation efficiency and ability to separate molecules based on their charge. It is generally a low cost and relatively easy analytical system to operate, although narrow bore capillaries present special handling problems. Nevertheless, CZE represents a breakthrough in high resolution, low flow-rate separations systems. Flow-rates essentially due to electroosmotic flow are typically in the range of 0.01–0.1 μl/min. With respect to combining CZE with mass spectrometry, these flow-rates are too low for stable operation of most ion sources and make-up liquid needs to be added to bring the flow-rate up to approximately 2–5 μl/min. A typical instrumental arrangement for CZE–CF-FAB is shown in Fig. 9. An interface is necessary because of the differences in the flow-rates of the CZE apparatus and the CF-FAB and electrospray systems. The CF-FAB system requires 2–5 μl/min of solvent flow for optimal performance and this is achieved using make-up solvent from a reservoir in the interface assembly. The interface can be of two types; the liquid-junction interface where the CZE capillary and the CF-FAB capillary are essentially butted up end-to-end in the interface [3], and the coaxial interface where the CZE capillary is threaded through the CF-FAB capillary almost to the target tip on the probe [10]. Each system has particular advantages and disadvantages. Basically, the liquid-junction interface is easier to set up and operate but gives significant band broadening. The coaxial interface provides higher resolution by giving minimal peak broadening but is mechanically more difficult to set up and run reproducibly.

The ion electropherograms for the analysis of 25 pmol of the tryptic digest of β-lactoglobulin A obtained by CZE–CF-FAB-MS (liquid-junction interface) is given in Fig. 10. The upper panel in the figure shows the total ion recording and the lower

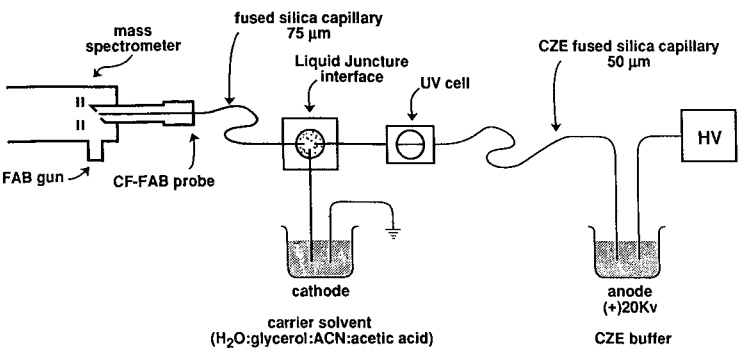

Fig. 9. The CZE–CF-FAB instrumental arrangement.

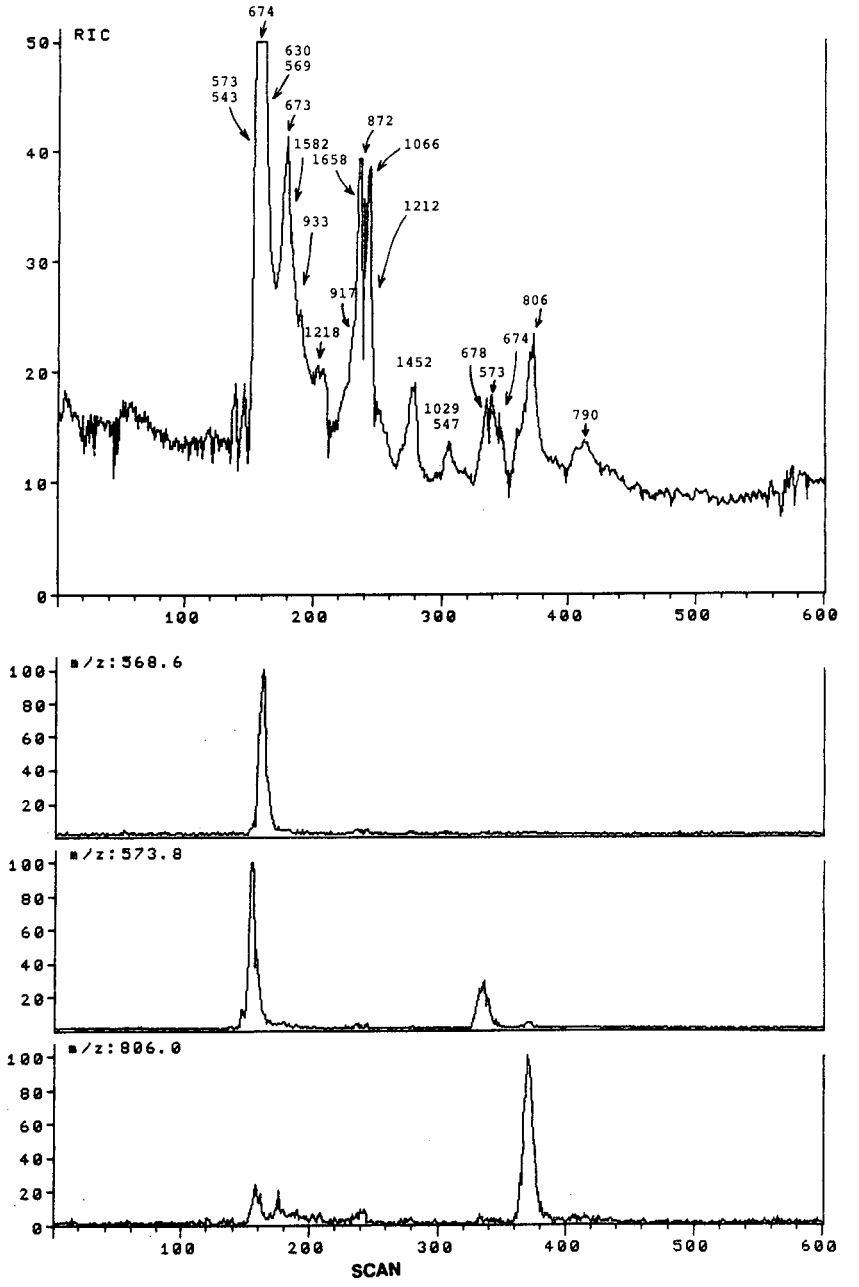

Fig. 10. The CZE–CF-FAB-MS analysis of the tryptic digest of 25 pmol of β-lactoglobulin A. (Top) the total ion recording using a scan range of m/z 500–2000, and (bottom) the selected ion recordings for three of the peptides.

panel several selected ion recordings. It is noted that the selected ion recording of m/z 573 shows two eluting peaks; the first is for the major tryptic fragment T7 and the second, unexpected in this analysis, for one or more peptides from several possible positions in the protein, produced by a contaminating protease. High-resolution separations were not used in this particular analysis because of the scan time required by the mass spectrometer (as discussed below). Nevertheless, specific peptides were easily identified in the mixture.

An important disadvantage of the use of a scanning detector such as a magnetic or quadrupole mass spectrometer is the fact that it cannot take full advantage of the extremely high-resolution separations achievable by CZE. For example, if one is scanning a decade of mass (300–3000 mass units), scan speeds of about 12–15 s would be required to get adequate signal-to-noise recordings for these spectra. This relatively lengthy scan time thus precludes the analysis of peaks in a separation process where the half-width of the peak emerging from the capillary is less than the scan time of the detector. Although there are integrating detectors and mass spectrometer analyzers that are becoming available that may be better suited for this purpose, these are, at the present time, either very expensive (array detectors) or are in development, such as the ion-trap detector. Despite this problem, the on-line combination of CZE with mass spectrometry provides a capability that will be invaluable for the structural analysis of proteins.

CONCLUSION

The chromatographic process limits the number of compounds entering the ion source of the mass spectrometer during the analysis. This has several important advantages. First, it can provide a temporal separation of compounds that have the same nominal mass or whose spectra have major ions in common. Second, it minimizes ion suppression effects, *i.e.*, the process whereby one or more compounds will not form ions as a result of the presence of other compounds in the mixture. Third, it allows the analyst to collect fractions and isolate purified compounds in the mixture for further study.

The two mass spectrometric ionization methods utilized in this work, CF-FAB and electrospray, have a number of features in common. They both have about the same sensitivity (5–50 pmol) when full-scan spectra are required for peptide digests analyzed by LC–MS methods and give optimal performance at flow-rates of about 5 μl/min. However, there are some important differences as well. Electrospray produces multiply-charged molecular species, an advantage that permits the analysis of molecules of significantly higher molecular weight than the upper mass limit of the spectrometer. CF-FAB gives high intensity $(M+H)^+$ ions, good MS–MS spectra, and tolerates a wide range of buffers with respect to their chemical diversity and volatility.

With respect to the use of microbore *versus* capillary bore LC columns, we were not able to discern significant differences in the analyses performed in this work. The identification of peptides in the mixtures could be accomplished using either column. The choice of one over the other may also be made based on other factors whose importance can vary depending on the task, *e.g.*, choice of flow-rates, use of a split or splitless interface, durability of the column, etc.

CZE–MS is potentially an extremely useful separation–detection device be-

cause its high-resolution capability can provide a new tool for the high sensitivity analysis needed for trace compound analysis. Nevertheless, the high-resolution process can also be a major problem if the mass spectrometer must scan a wide mass range. Currently, the CZE–MS combination is most effectively used for target compound analysis or in applications where only narrow mass ranges need to be scanned.

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Purification of canine prolactin and growth hormone by fast protein liquid chromatography

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ABSTRACT

Prolactin and growth hormone are two peptide hormone with a very similar structure. A simple method is described for the simultaneous purification of these two peptides from canine pituitary extract by fast protein liquid chromatography. After extraction at pH 5.0 and 9.6 and anion-exchange chromatography on a MonoQ column, prolactin and growth hormone are then separated by gel filtration chromatography on two Superose columns coupled in series. The different fractions of the purification scheme are checked for the presence of the peptide hormones by sodium dodecyl sulphate gel electrophoresis in the Pharmacia PhastSystem. Each hormone is also characterized by its behaviour in a Western Blotting Detection System.

INTRODUCTION

Since the dog is a very useful model in medical research, canine prolactin (cPRL) and canine growth hormone (cGH) have been purified by several authors in order to prepare the components of radioimmunoassays used in the study of plasma levels of these hormones in various physiological states [1–6]. The development of fast protein liquid chromatography (FPLC) has allowed the rapid separation of complex mixtures of biological molecules [7–8]. We have applied this technique to purifiy cPRL and cGH from canine pituitary glands simultaneously, in only three steps.

EXPERIMENTAL

Equipment

The FPLC system (Pharmacia, Uppsala, Sweden) consisted of an LCC-500 liquid chromatography controller, two P-500 pumps, an MV-7 injection valve and a FRAC 100 fraction collector. The protein content of the eluent was analysed on a UV-M monitor (280 nm) and recorded with a two-channel REC-482 recorder. The anion-exchange column was a Mono Q HR 5/5 prepacked column. A Superose 6 HR 10/30 column was coupled in series with a Superose 12 HR 10/20 column for the gel filtration chromatography.

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Polyacrylamide gel electrophoresis and Western blotting

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on PhastGel gradient 10–15 (Pharmacia). Samples treated with SDS and β-mercapthoehanol were processed in the Pharmacia PhastSystem and the gels were developed with the Coomassie staining procedure [9].

After electrophoresis, the proteins were transferred over 30 min on a nitrocellulose membrane (LKB) with the PhastTransfer semi-dry transfer kit (Pharmacia). Bound proteins were detected by incubation with an antiserum against ovine PRL (BioScience) or an antiserum against bovine GH (Ventrex) and a secondary antibody conjugated to alkaline phosphatase (Immum-Blot kit, Bio-Rad).

Purification procedure

A total of sixteen pituitaries were removed from Beagle dogs within 2 h of death and kept frozen until required. The glands were sonicated in 20 ml of 50 m*M* ammonium acetate buffer, pH 5.5 at 4°C in a Soniprep 150 (MSE). The homogenate was first extracted in buffer pH 5.5. After centrifugation, the pellet was resuspended in 20 m*M* piperazine, pH 9.6 and extracted overnight. After centrifugation at 27 000 *g* at 4°C for 30 min, the supernatant was removed to be processed by anion-exchange chromatography.

A total of 10 ml of supernatant were injected on a Mono Q HR 5/5 column. The separation was carried out with an elution gradient from 20 m*M* piperazine, pH 9.6 to

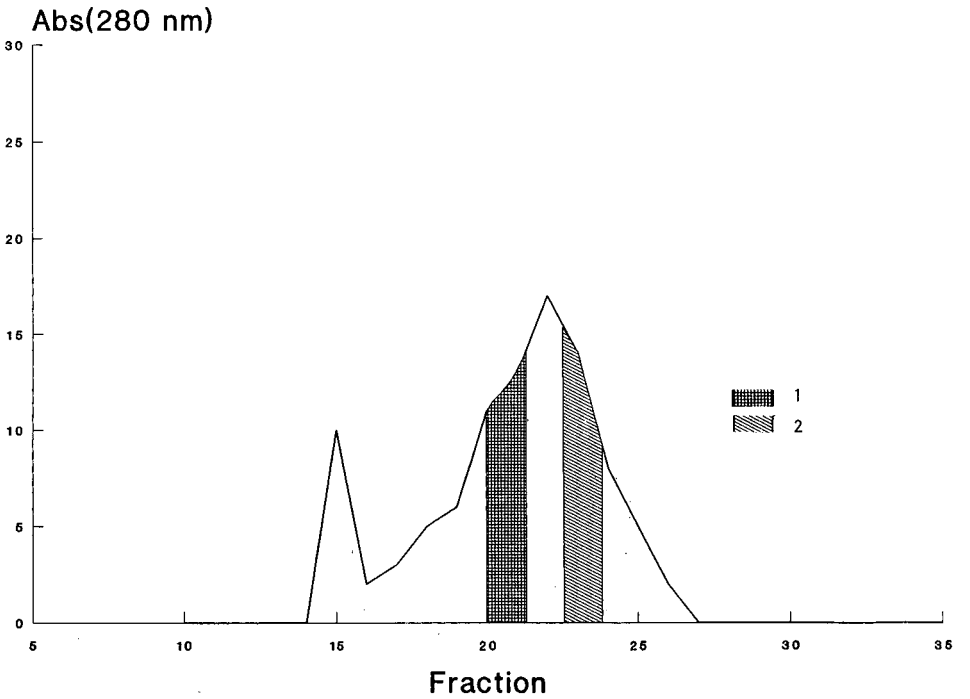

Fig. 1. Gel filtration chromatography on a series of Superose 12 HR 10/30 and Superose 6 HR 10/30 columns. (-) Absorbance at 280 nm; 1 = cPRL fractions; 2 = cGH fractions.

20 m*M* piperazine–1 *M* sodium chloride pH 9.6. The buffer was chosen according to the methods of Stanton *et al.* [10] on ovine prolactin. The segmented gradient started 5 min after injection with a flow-rate of 1 ml/min: 0–5 min, 0% NaCl; 5–25 min, 0–20% NaCl (linear); 25–30 min, 20% NaCl; 30–40 min, 20–40% NaCl (linear); 40–45 min, 40% NaCl; 45–55 min, 40–100% NaCl (linear); 55–60 min, 100% NaCl with a flow-rate of 1 ml/min. The 1-ml fractions were analysed for the presence of cPRL and cGH by SDS-PAGE.

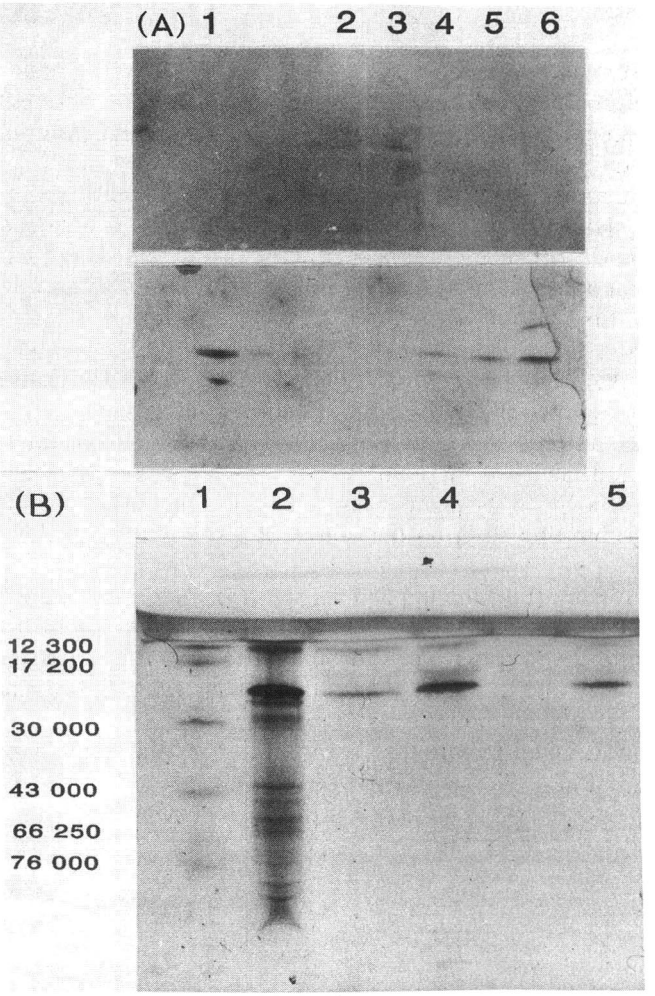

Fig. 2. (A) Western blotting of the fractions obtained after gel filtration chromatography. The membrane on top was incubated with an antiovine PRL serum while the membrane below was incubated with an antibovine GH serum. Lanes: 1 = pool of the fractions obtained after anion-exchange chromatography (not applied on the first membrane); 2–6 = fractions 20–24 respectively. (B) SDS-PAGE of the fraction obtained during the purification process. Lanes: 1 = molecular weight marker (LKB); 2 = extract of canine pituitary glands S2; 3 = cGH obtained after Superose gel filtration; 4 = pool of the fraction obtained after Mono Q anion-exchange chromatography; 5 = cPRL obtained after Superose gel filtration.

The fractions from the Mono Q column containing cPRL and cGH were pooled and concentrated overnight on Bio-Gel concentrator resin (Bio-Rad). The concentrated fraction (1 ml) was then injected on Superose 12 HR 10/30 and Superose 6 HR 10/30 coupled in series and eluted with a solution of 20 m*M* piperazine, pH 9.6 at a flow-rate of 0.5 ml/min. The 35 fractions of 1 ml obtained were checked by SDS-PAGE and Western blotting. The pure fractions obtained after gel filtration chromatography were dialysed overnight and lyophilised.

RESULTS AND DISCUSSION

The advantage of the extraction procedure used is the removal of the major blood proteins [5] and therefore the improvement of the resolution on the Mono Q HR 5/5 column. During anion-exchange chromatography, cPRL and cGH elute together as a large peak between 140 and 200 m*M* NaCl.

The resolution of the fractions from the Mono Q on the two columns, Superose 6 HR 10/30 and Superose 12 HR 10/30 is shown in Fig. 1. A large peak is obtained between fractions 17 and 27. On the membrane incubated with the antiovine PRL serum, one band is seen for fractions 20 and 21 (lanes 2 and 3) (fig. 2A). On the second membrane, incubated with antibovine GH serum, only fractions 22 to 24 react (lanes 4–6). According to these results, fractions 20 and 21 containing cPRL are pooled, fraction 22 is kept apart and the cGH-rich fractions 23 and 24 are pooled. The purity of the two hormone batches is shown by SDS-PAGE (Fig. 2B, lanes 3 and 5).

Only one band for cPRL and one band for cGH are observed after gel filtration chromatography. The low-molecular-weight components which appear on SDS-PAGE are degradation products due to the proteolysis of the hormones. These bands are also detected by Western blotting showing the immunological activity of these components (not shown here).

The technique of the Western blotting applied on selected fractions obtained after each chromatographic step can be very useful in checking the resolution of the pituitary hormones. The use of the PhastSystem and of the PhastTransfer semi-dry transfer kit allows the analysis of the fractions in less than 2 days while 5 days are necessary to have the results of the radioimmunoassay.

This procedure of purification of the pituitary hormones allows the recovery of an average of 258 μg of cPRL and of 735 μg of cGH (Table I).

TABLE I

PURIFICATION TABLE

Stage	Concentration proteins (mg/ml)	Total proteins (mg)	Yield (%)
Crude extract	3.34	53.5	
Extraction	0.43	5.2	9
Ion-exchange	0.42	2.5	48
Gel filtration			
PRL fraction	0.13	0.3	10
GH fraction	0.24	0.7	29

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Advantages and limitations of pre-column derivatization of amino acids with dabsyl chloride

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ABSTRACT

This paper describes a procedure for the derivatization of amino acids using dabsyl chloride and the high-performance liquid chromatographic determination of the resulting dabsyl-amino acids. The dabsyl derivatives have a number of advantages over other derivatives, including a simple derivatization procedure, very good stability, good reproducibility and a good limit of detection for the method, complete high-performance liquid chromatographic separation of all the amino acids and specific detection at a wavelength in the visible region. The derivatization parameters were optimized to obtain a fast and practical detection method. The method presented here has been applied to a large number of different projects and an example is given for each application.

INTRODUCTION

Several pre-column derivatization methods have been reported for the determination of amino acids using high-performance liquid chromatography (HPLC). These methods use derivatization with reagents such as phenylisothiocyanate [1,2], dansyl chloride [3,4], *o*-phthalaldehyde [5,6], dabsyl chloride [7–11] and 9-fluorenylmethylchloroformate [12]. After extensive testing of several pre-column derivatization methods, it was found that the derivatizations with dabsyl chloride [13] and with 9-fluorenylmethylchloroformate [14] gave the best results, especially with respect to the stability and consequently the reproducibility of the derivatives. The amino acid derivatives of phenylthiohydantoin [15] and especially of *o*-phthalaldehyde were found to be unstable.

This paper describes a procedure for the derivatization of amino acids using dabsyl chloride and the HPLC determination of the resulting dabsyl-amino acids. A number of applications in which amino acids are involved are given.

EXPERIMENTAL

Chemicals

The solvents used were obtained from Merck (Darmstadt, Germany). Dabsyl chloride and the amino acid standards were obtained from Pierce Europe (Oud

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Beijerland, The Netherlands). The amino acids were dissolved in 0.05 mol/l hydrochloric acid and stored at −20°C before use.

HPLC equipment

The HPLC equipment consisted of the following components: an automatic injector (Model 231, Gilson, Meyvis, Bergen op Zoom, The Netherlands); two solvent delivery systems (Model 2150, LKB, Woerden, The Netherlands) controlled by a gradient controller (Model 2152, LKB); a UV–visible detector (Model 200, Linear, Analytical, Maasdijk, The Netherlands); and a computing integration system (Model CI-10B, LDC/Milton Roy, Interscience, Breda, The Netherlands).

Derivatization

The derivatization reagent consisted of a solution of 4 nmol/l 4-dimethylaminoazobenzene-4′-sulfonyl chloride (DABS-Cl) in acetonitrile. This solution was prepared daily. Acetonitrile (40 μl) and dabsyl chloride reagent (40 μl) were added to 20 μl of the standard solution. For each sample, 100 μl of sample were added to 100 μl of dabsyl chloride reagent. The mixtures were heated for 10 min at 70°C in an oven. A 900-μl volume of a solution of sodium hydrogenphosphate (0.1 mol/l, pH 7.0)–acetonitrile–methanol (70:20:10, v/v/v) was added to the reaction mixture. A 20-μl volume of this mixture was injected on to the HPLC column.

Sample preparation and HPLC conditions

Determination of standard amino acids. A standard solution of amino acids (39 mmol/l) was diluted five-fold in 0.05 mol/l hydrochloric acid. To 20 μl of the diluted standard solution, 980 μl of sodium hydrogen carbonate (0.05 ml/l) were added. After derivatization, 20 μl of the resulting solution were injected on to the HPLC system. The HPLC column consisted of two cartridges (100 × 3.0 mm) in a Chromsep holder with Hypersil ODS or another reversed-phase material such as Chromspher C_{18}, Spherisorb ODS-2, Nucleosil C_{18} or Zorbax ODS (Chrompack, Middelburg, The Netherlands). For all other applications an HPLC column with an I.D. of 4.6 mm was used. The column was thermostated at 30°C. Mobile phase A consisted of 25 mmol/l sodium acetate (pH 6.5)–acetonitrile–methanol (70:20:10, v/v/v). Mobile phase B consisted of 25 mmol/l sodium acetate (pH 6.5)–acetonitrile–methanol (10:45:45, v/v/v). The elution was performed as follows: from 0 to 20 min, a linear gradient from 0 to 50% B; from 20 to 27 min, a linear gradient from 50 to 100% B; from 27 to 32 min, 100% B. The flow-rate was 1 ml/min. The UV–visible detector was adjusted to 456 nm.

Determination of glycine in preparations of anti-thymocyte globulin. A 25-μl volume of a solution with valine (0.3 mol/l) as an internal standard, 55 μl of 0.1 mol/l hydrochloric acid and 1900 μl of sodium hydrogencarbonate buffer (0.05 mol/l) was added to 20 μl of the preparation of anti-thymocyte globulin. A 50-μl volume of this solution was added to 150 μl of acetonitrile to precipitate the proteins. A 100-μl volume of the supernatant was used in the derivatization procedure. After derivatization, 20 μl were injected on to the HPLC system. The HPLC column (150 × 4.6 mm) was packed with Hypersil ODS (5 μm). The mobile phase consisted of 25 mmol/l sodium acetate (pH 6.5) and acetonitrile (72:28, v/v). The elution was performed isocratically from 0 to 15 min. The column was then cleaned of the tightly bound components with 100% methanol for 2 min. The flow-rate was 1 ml/min at room temperature. The UV–visible detector was adjusted to 436 nm.

Determination of amino acids in cell culture media. A 200-μl volume of acetonitrile was added to 100 μl of a sample of culture medium to precpitate the proteins. A 40-μl volume of the supernatant was used in the derivatization procedure, to which 40 μl of dabsyl reagent were added. After heating, 240 μl of a mixture of phosphate buffer (0.05 mol/l, pH 7.0) and methanol (1:1) were added. From this mixture, 20 μl were injected on to the HPLC system. The HPLC column (150 × 4.6 mm) was packed with Hypersil ODS (5 μm) and was thermostated at room temperature. Mobile phase A consisted of 100 mmol/l sodium acetate (pH 6.5)–acetonitrile–methanol (56:16:28, v/v/v). Mobile phase B consisted of 100% methanol. The elution was performed as follows: from 0 to 5 min, 0% B; from 5 to 30 min, a linear gradient from 0 to 40% B; from 30 to 35 min, 40% B; from 35 to 40 min, a linear gradient from 40 to 100% B; and from 40 to 43 min, 100% B. The flow-rate was 1 ml/min. The UV–visible detector was adjusted to 436 nm.

Determination of hydrophobic amino acids in human serum. A 400-μl volume of acetonitrile was added to 200 μl of a serum sample to precipitate the proteins. A 40-μl volume of the supernatant was used in the derivatization procedure, to which 40 μl of dabsyl reagent were added. After heating, 70 μl of a mixture of phosphate buffer (0.05 mol/l, pH 7.0) and methanol (1:1) were added. From this mixture, 20 μl was injected on to the HPLC system. The HPLC column (150 × 4.6 mm) was packed with Hypersil ODS (5 μm). Mobile phase A consisted of 100 mmol/l sodium acetate (pH 6.5)–acetonitrile–methanol (56:16:28, v/v/v). Mobile phase B consisted of 25 mmol/l sodium acetate (pH 6.5)–acetonitrile (71:29, v/v). The elution was performed as follows: from 0 to 16 min, 0% B; from 16 to 17 min, a linear gradient from 0 to 100% B; and from 17 to 35 min, 100% B. The flow-rate was 1 ml/min at room temperature. The UV–visible detector was adjusted to 436 nm.

Determination of tyrosine and thyronine derivatives. A 200-μl volume of acetonitrile was added to 100 μl of sample of thyroid tissue to precipitate the proteins. A 100-μl volume of the supernatant was used in the derivatization procedure, to which 100 μl of dabsyl reagent were added. After heating, 400 μl of a mixture of phosphate buffer (0.05 mol/l, pH 7.0) and methanol (1:1) were added. From this mixture, 20 μl were injected on to the HPLC system. The HPLC column (150 × 4.6 mm) was packed with Hypersil ODS (5 μm). Mobile phase A consisted of 25 mol/l sodium acetate (pH 6.5)–methanol (44:56, v/v). Mobile phase B consisted of 100% methanol. The elution was performed as follows: from 0 to 15 min, a linear gradient from 20 to 65% B; from 15 to 18 min, 65% B; from 18 to 19 min, a linear gradient from 65 to 100% B; and from 19 to 21 min, 100% B. The flow-rate was 1 ml/min at room temperature. The UV–visible detector was adjusted to 436 nm.

RESULTS

Derivatization

The pH dependence of the derivatization procedure was tested for five amino acids: Glu, Ala, Val, Leu and Lys. Between pH 7.5 and 9.0 no significant differences were observed in either the relative or absolute peak heights. The time and temperature dependence of the derivatization was investigated in the same experiment. In the initial experiments the derivatization was stopped by a ten-fold dilution of the mobile phase of the HPLC procedure. At derivatization times longer than 15 min, a decrease in the

peak heights was observed, even at room temperature. Therefore, in all further experiments the derivatization was stopped by the addition of ammonia to remove the excess of dabsyl chloride. In all experiments the HPLC peak heights were compared with those obtained at 70°C for 15 min. The derivatization at 20°C was complete after only 30 min for the following amino acids: Gly, Ile, Val, Leu, Lys, Met, Phe, Pro and Trp. For five amino acids, Asn, Cys, Ser, His and Gln, the peak heights were even higher than those obtained by derivatization at 70°C (data not shown). Almost the same derivatization behaviour was obtained at 37°C (see Table I). At 50°C the derivatization of most of the amino acids was complete after 30 min. At 70°C, all twenty amino acids showed a derivatization behaviour which was a function of time. The optimum time of derivatization at 70°C was 15–30 min.

As the amino acid standards were dissolved in dilute hydrochloric acid, the influence of the concentration of chloride ions was investigated. An increase from 50 to 150 mmol/l chloride ions has an effect on the derivatization behaviour of the amino acids (Table II).

Most of the peak heights decreased in intensity to a maximum of 15% of the original value as the chloride ion concentration increased. A few amino acids showed an increase in peak height to a maximum value of 30% (*e.g.*, Ile).

TABLE I

EFFECTS OF TEMPERATURE AND TIME OF DERIVATIZATION OF AMINO ACIDS WITH DABSYL CHLORIDE

The peak heights of the dabsyl derivatives are shown after various derivatization times (5, 10, 15, 30 and 60 min) at 37, 50 and 70°C. The data are presented as a percentage of the standard condition (15 min at 70°C).

Amino acid	Derivatization time at 37°C (min)					Derivatization time at 50°C (min)					Derivatization time at 70°C (min)				
	5	10	15	30	60	5	10	15	30	60	5	10	15	30	60
Asp	28	46	59	84	97	48	72	83	93	101	73	98	100	104	108
Asn	46	72	88	112	116	61	85	94	106	114	83	96	100	102	108
Gly	100	100	103	103	99	99	99	100	98	102	115	103	100	104	102
Ile	98	104	99	88	78	104	101	98	95	98	115	101	100	97	97
Cys	24	59	85	129	149	70	99	120	136	139	26	92	100	105	111
Glu	41	67	79	102	113	62	88	95	105	102	78	99	100	105	102
Ala	69	89	99	105	102	80	90	96	97	110	89	98	100	102	99
Val	89	101	100	98	90	93	97	98	96	109	95	99	100	98	99
Leu	83	100	101	102	93	90	95	99	97	109	98	105	100	105	103
Lys	64	95	102	109	93	86	94	96	96	110	94	102	100	105	107
Ser	66	93	108	116	139	75	95	102	108	106	83	103	100	106	107
Met	103	123	130	126	131	97	106	104	101	101	97	102	100	101	100
Phe	91	104	107	104	111	91	97	100	99	100	93	101	100	105	100
His	44	79	101	47	13	63	96	109	116	118	73	102	100	108	101
Gln	57	86	94	111	109	71	92	92	104	103	81	92	100	100	96
Pro	103	101	98	99	96	98	97	93	99	97	98	98	100	103	96
Tyr	15	30	41	53	59	45	76	74	91	91	66	96	100	104	99
Thr	54	81	92	102	103	67	84	96	101	103	46	99	100	104	108
Arg	53	81	91	104	109	68	89	97	103	105	42	98	100	102	103
Trp	112	114	107	103	99	111	107	106	107	108	80	108	100	108	106

TABLE II

EFFECT OF THE CONCENTRATION OF SODIUM CHLORIDE ON THE DERIVATIZATION OF AMINO ACIDS WITH DABSYL CHLORIDE

The data are shown as a percentage of the standard condition (15 min at 70°C with a concentration of sodium chloride of 50 mmol/l).

Amino acid	Concentration of NaCl (mmol/l)			
	50	75	100	150
Asp	100	93	97	91
Asn	100	92	90	87
Gly	100	98	95	90
Ile	100	104	120	131
Cys	100	107	112	108
Glu	100	103	102	99
Ala	100	107	103	101
Val	100	106	100	97
Leu	100	115	115	115
Lys	100	98	92	86
Ser	100	87	92	85
Met	100	93	96	88
Phe	100	115	108	109
His	100	94	92	89
Gln	100	102	96	96
Pro	100	101	91	90
Tyr	100	99	87	84
Thr	100	102	100	97
Arg	100	98	93	85
Trp	100	114	109	115

Characterization of dabsyl derivatives

The dabsyl derivatives of the various amino acids show absorption maxima ranging from 448 to 468 nm. The individual data are summarized in Table III, together with the relative intensities of the dabsyl derivatives. Three amino acid derivatives show a substantially higher signal than the remainder, which is a result of, the occurrence of a double-substituted dabsyl moiety on the side-chain amino group (Lys), the phenolic hydroxyl group (Tyr), and the NH of the imidazole moiety (His). Low intensities are observed for Gln and Cys.

The stability of the dabsyl amino acid derivatives is very good. The HPLC signal of five derivatized amino acids (Tyr, Val, Ile, Trp and Phe) was followed for 7 days. The average signal height was 97.2% after 1 week. The limit of detection of the dabsylated amino acids is also very good. An average value of 100 pg (about 1 pmol) was obtained with Ser, Ala, Pro and Cys with a signal-to-noise ratio of 3 using the Linear 200 variable-wavelength detector with a wolfram lamp at 456 nm.

High-performance liquid chromatographic separations

To obtain the best separation of all twenty naturally occurring amino acids, combinations of methanol, tetrahydrofuran and acetonitrile were tested within a 25 mmol/l sodium acetate buffer as the aqueous phase. The pH of the buffer was varied

TABLE III

MAXIMUM WAVELENGTHS OF THE UV-VISIBLE SPECTRUM AND RELATIVE INTENSITIES OF PEAK AREAS (RELATIVE TO Ala = 100%) OF TWENTY NATURALLY OCCURRING AMINO ACIDS

Amino acid	Maximum wavelength (nm)	Intensity (%)
DABS-OH	456	–
Trp	462	83.2
Thr	464	88.8
Ser	464	96.4
Pro	464	108.0
Phe	458	92.8
Met	464	80.5
Lys	448	203.8
Asn	464	56.4
Arg	464	83.0
Ala	464	100
Tyr	456	273.8
Leu	462	94.2
Ile	464	90.5
Glu	468	82.7
Cys	450	32.5
Gly	464	100.0
His	458	134.2
Val	462	87.7
Asp	466	85.0
Gln	468	52.3

from 5.5 to 7.5; the best result was obtained with the acetonitrile–methanol buffer at pH 6.5. Attempts to use chemometric models for the calculation of the best separation failed because of the unusual parabolic behaviour of the amino acids which occurred as the percentage of methanol and acetonitrile was varied. This is shown in Fig. 1, where mobile phase B was acetonitrile and A was methanol. The effect of small amounts of

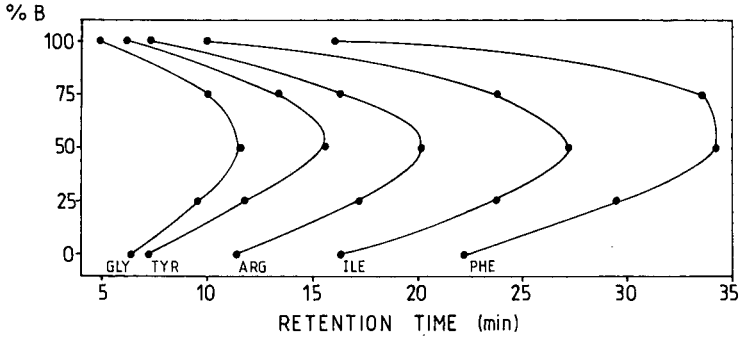

Fig. 1. Retention time behaviour of dabsylated amino acids as a function of solvent composition. Solvent A is methanol and solvent B is acetonitrile (A + B = 100%).

other organic modifiers such as isopropanol, dimethylformamide and triethylamine was also tested. On the addition of 1% isopropanol, only small changes were observed, especially in the region Pro–Val–Arg. The addition of 1% dimethylformamide or triethylamine resulted in poorer separations; an increase in the buffer strength from 25, to 100 mmol/l sodium acetate did not improve the separation. All the amino acids moved to longer retention times, except Arg.

The optimum conditions were: a biphasic linear gradient from 25 mmol/l sodium acetate (pH 6.5)–acetonitrile–methanol (70:20:10, v/v/v) to 25 mol/l sodium acetate pH 6.5)–acetonitrile–methanol (10:45:45, v/v/v), as described under Experimental. Under these conditions five different reversed-phase packing materials were tested in commercially available cartridges of 20 cm length; these were Hypersil ODS, Chromspher C_{18}, Spherisorb ODS-2, Nucleosil C_{18} and Zorbax ODS. The retention times are summarized in Table IV. In Fig. 2 it is shown that under the experimental conditions described, the best separation is achieved with Zorbax ODS. As the di-substituted derivatives of His and Tyr are low in intensity, the peaks marked with an asterix in Fig. 2 may originate from the mono-substituted analogues.

TABLE IV

RETENTION TIME BEHAVIOUR OF ALL TWENTY NATURALLY OCCURRING AMINO ACIDS ON CARTRIDGES PACKED WITH DIFFERENT REVERSED-PHASE MATERIALS

Experimental conditions are as described in the text.

Amino acid	Retention time (min)				
	Nucleosil C_{18}	Zorbax ODS	Chromospher C_{18}	Spherisorb ODS-2	Hypersil ODS
Trp	17.39	17.47	14.43	14.87	15.93
Thr	11.17	11.11	8.57	8.73	9.77
Ser	10.49	10.35	7.97	8.07	9.15
Pro	14.13	13.63	11.29	11.57	12.37
Phe	18.29	18.47	15.49	15.77	16.69
Met	14.79	15.57	12.59	12.27	13.79
Lys	26.85	26.77	25.29	25.61	25.83
Asn	9.21	8.93	6.81	6.87	7.89
Arg	13.47	12.19	11.27	12.19	11.65
Ala	12.17	11.95	9.53	9.55	10.49
Tyr	28.29	28.27	27.13	27.25	27.35
Leu	16.91	17.01	14.35	14.35	15.21
Ile	16.37	16.49	13.77	13.75	14.61
Glu	4.81	5.19	3.15	2.63	4.07
Cys	21.69	22.05	19.23	18.33	20.21
Gly	11.87	11.49	9.29	9.21	10.25
His	27.53	27.53	26.23	26.33	26.67
Val	14.29	14.39	11.67	11.69	12.65
Asp	4.27	4.81	2.93	2.47	3.81
Gln	10.03	9.85	7.69	7.57	8.97

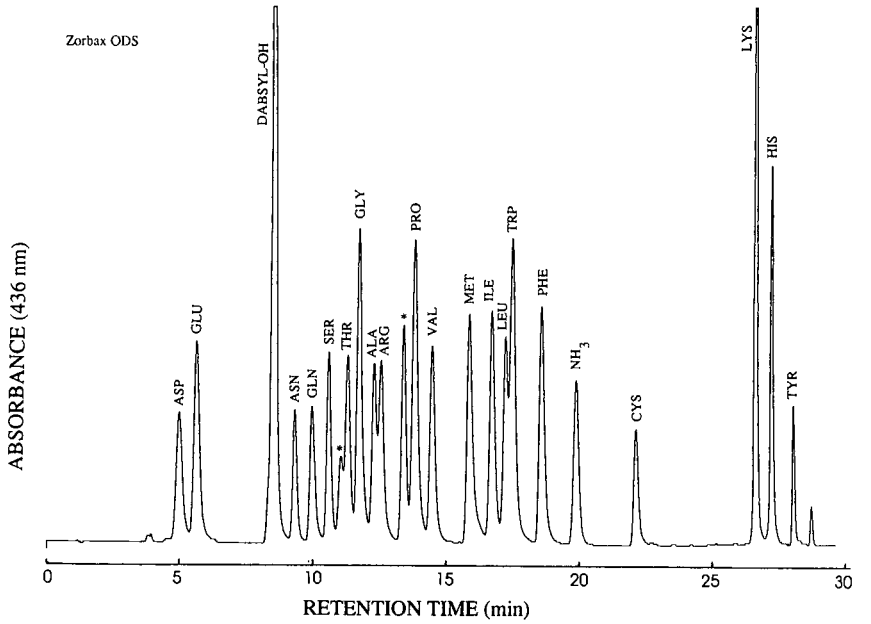

Fig. 2. Chromatogram of the separation of the dabsyl derivatives of all twenty naturally occurring amino acids on a cartridge of Zorbax ODS. The elution conditions are described in the text. The peaks indicated with an asterix have not been identified.

Applications

In our institute, the quantitative determination of amino acids by dabsylation followed by HPLC determination has been used in a number of applications. A brief description and an example are given for each application.

The characterization of proteins and peptides obtained by chromatographic or electrophoretic isolation procedures can be carried out by sequencing or total amino acids analysis. In Fig. 3 the HPLC profile is shown for dabsyl amino acids after the acid hydrolysis of bovine serum albumin. It should be noted that Gln and Asn are respectively converted to the corresponding amino acids Glu and Asp. Amino acid analysis after hydrolysis is used in our institute to support mass spectrometric sequencing measurements and to control the composition of synthetic peptides.

Another application is the off-line monitoring of the consumption of essential amino acids in cell culture media. In the culture of animal cells for the production of monoclonal antibodies, the concentration of ten amino acids was followed. Fig. 4 shows HPLC profile of a typical amino acid mixture taken from the culture medium, which has been optimized for this application.

The dabsylation of amino acids is used also for the control of buffer components in preparations of anti-thymocyte globuline, which is produced by our institute. In these preparations glycine must be identified and determined; this is carried out using valine as an internal standard.

Another study in which the dabsylation of amino acids was used to determine amino acids was directed to the problem of premenstrual complaints. One of the most

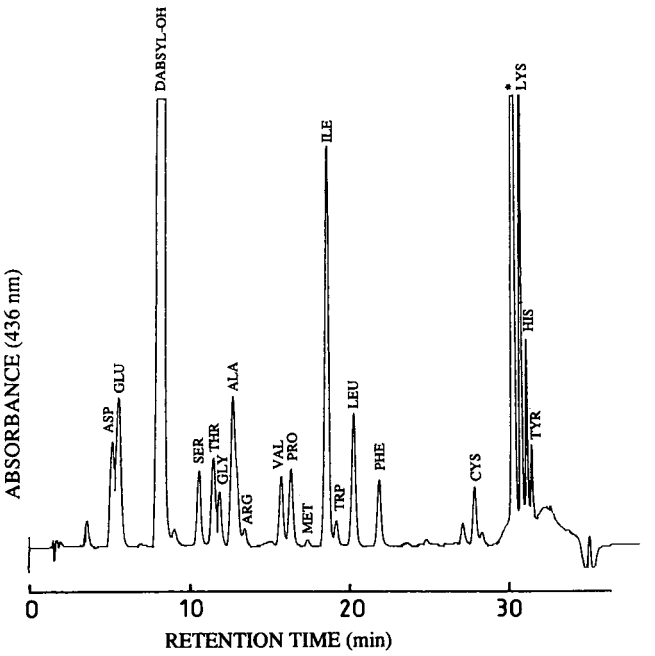

Fig. 3. Chromatogram of the separation of the dabsyl derivatives of amino acids from an acid hydrolysate of bovine serum albumin. The elution conditions are described in the text.

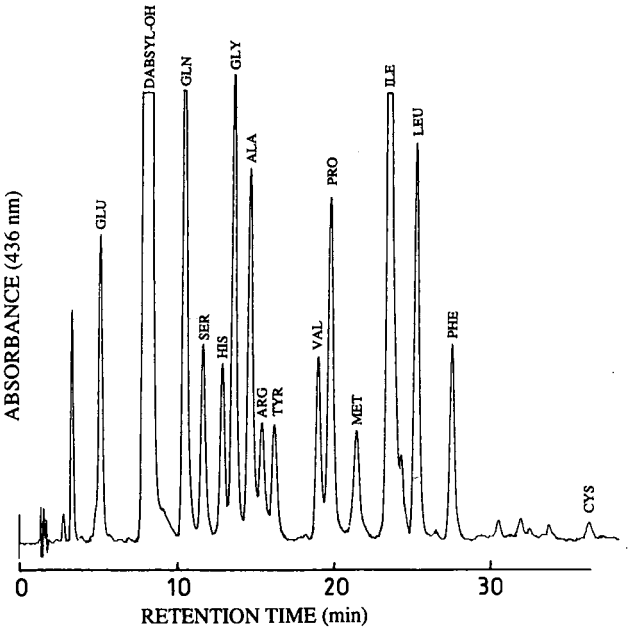

Fig. 4. Chromatogram of the separation of the dabsyl derivatives of amino acids from a cell culture medium. The elution conditions are described in the text.

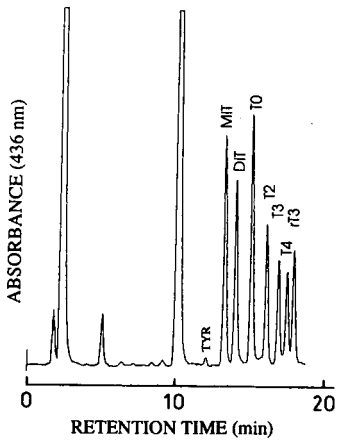

Fig. 5. Chromatogram of the separation of the dabsyl derivatives of a number of thyronine and tyrosine analogues found in the thyroid. The elution conditions are described in the text.

important parameters in this study was the ratio in serum between Trp and other hydrophobic amino acids such as Val, Ile, Leu and Phe. This ratio determines the amount of TRP which enters the brain via the blood–brain barrier. As Trp is considered to be the precursor of serotonin in the brain, the determination of the ratio of Trp and the other hydrophobic amino acid in serum is an important parameter for premenstrual complaints. The results of this work will be presented elsewhere [16].

The thyroid is an important organ which produces thyroid hormones, such as triiodothyronine (T3), reversed triiodothyronine (rT3) and tetraiodothyronine (T4). These hormones are synthesized in the thyroid follicle by the coupling of precursor hormones, such as tyrosine, monoiodotyrosine (MIT) and diiodothyrosine (T2). In toxicology the relative amounts of these hormones and precursors are an important parameter in assessing the toxicity of xenobiotic agents. As these compounds are essentially amino acids, the determination *via* dabsylation was investigated. For all components di-substituted dabsyl derivatives were formed which could be separated and quantitated by HPLC (Fig. 5).

DISCUSSION

The derivatization of amino acids with dabsyl chloride gives the best results at 70°C for 15–30 min, as was described previously [9,10]. Neither the pH nor the time of derivatization is very critical. The only restriction is with derivatizations performed at temperatures lower than 70°C. For some amino acids an incomplete derivatization was observed at such temperatures. In addition, it was observed that at lower temperatures some amino acids show higher peak heights than at higher derivatization temperatures. This might indicate that the dabsyl amino acids derivatized at 70°C are tending to decrease in intensity as a result of instablity. Therefore the conditions of incubation should be standardized at 70°C as far as possible. It was also observed that the concentration of chloride ions should be kept constant and preferably as low as possible.

The derivatives were characterized by a good stability, a good limit of detection and a maximum absorbance at 448–468 nm. At this wavelength no or little interference is expected from matrix components. The separation of all twenty naturally occurring amino acids can be performed by reversed-phase gradient HPLC within 30 min. The best mobile phase was a mixture of methanol and acetonitrile in a sodium acetate buffer at pH 6.5. Several reversed-phase materials were tested under identical experimental conditions. The best separation was observed with Zorbax ODS and Hypersil ODS.

The pre-column derivatization with dabsyl chloride has proved to be a very good and convenient method of determining amino acids. The determination of amino acids described here has already been applied for a great number of applications in our institute. The results of these applications will be the subject of further papers.

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CHROMSYMP. 2287

Comparison of high-performance liquid chromatographic detection methods for thyronine and tyrosine residues in toxicological studies of the thyroid

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ABSTRACT

Four high-performance liquid chromatographic methods for the detection of thyroid hormones (iodinated thyronines) and precursors (iodinated tyrosines) have been developed and evaluated. Two methods consist of direct determination of the parent compounds with detection at ultraviolet wavelength (230 nm) and with electrochemical detection. The two other methods consist of a pre-column derivatization (with fluorenylmethyl chloroformate and dabsyl chloride) prior to high-performance liquid chromatographic analysis. The various methods were evaluated based on their practical use and sensitivity. The method with direct ultraviolet detection turned out to be the most practical method. With this method analyses of thyroid homogenates have been performed from rats from a toxicological experiment.

INTRODUCTION

In the thyroid gland synthesis of the thyroid hormones 3,3′,5′-triidothyronine (T3) and 3,3′,5,5′-tetraiodothyronine (T4) takes place in the thyroidal follicle [1]. The formation of the precursors 3-monoiodotyrosine (MIT) and 3,5-diiodotyrosine (DIT) and the coupling of both precursors in the formation of T3 and T4 is controlled by thyroid peroxidase [2]. Thyroglobulin is the major storage of these hormones and the precursors.

The relative concentrations of these compounds are an important parameter for the study of toxicological effects on the thyroid [3,4]. In the present study, a number of detection methods have been developed for the thyroid hormones, precursors and metabolites using high-performance liquid chromatography (HPLC) [5–7]. Four HPLC methods will be compared: the determination of the parent compounds with UV and electrochemical detection and the determination of the compounds after precolumn derivatization with dabsyl chloride [8] and fluorenylmethyl chloroformate (FMOC-Cl) [9]. Results will be shown for a number of assay parameters such as limit of detection and practical applicability.

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EXPERIMENTAL

Materials

HPLC solvents were of analytical grade (Westburg, Leusden, The Netherlands). Water was taken from a Millipore Milli-Q filtration unit. Dabsyl chloride was obtained from Sigma, Brunschwig (Amsterdam, The Netherlands). FMOC-Cl and 1-adamantylamine were obtained from Aldrich (Brussels, Belgium). The following tyrosine and thyronine standards were used: tyrosine (TYR) obtained from Merck (Darmstadt, Germany), 3-MIT, DIT, thyronine (T0), 3,5-diiodothyronine (T2), T3, 3,3′,5-triiodothyronine (rT3) and T4, all obtained from Sigma.

HPLC with direct UV detection

The HPLC equipment consisted of the following components: an autoinjector (Varian Model 9000), two solvent delivery systems (Perkin Elmer Series 10), two low-pressure three-way valves (Rheodyne 5301), a high-pressure valve (Rheodyne 7010), a UV–VIS detector (Linear Model 200), a data acquisition system (Axxiom, Analytiça, Maasdijk, The Netherlands). The column system consisted of a cartridge of Chromspher C_{18} (100 × 3 mm I.D., 5 μm) with a concentrating column (15 × 3.2 mm I.D., 7 μm) (Brownlee Newgard RP-18, Inacom Instruments, Veenendaal, The Netherlands) and was maintained at 35°C with an electric column oven (LKB Model 2155). The following HPLC conditions were used: after injection the concentrating column was eluted for 2 min with mobile phase A (phosphate buffer, 0.05 *M*, pH 2.2, containing 2.5 m*M* heptanesulphonic acid). Then the analytical column was put in line with the concentrating column and eluted for 3.5 min with mobile phase C (a mixture of phosphate buffer, 0.05 *M*, pH 2.5, containing 2.5 m*M* heptanesulphonic acid–methanol, 65:35, v/v) and for 14.5 min with mobile phase D (a mixture of phosphate buffer, 0.05 *M*, pH 2.5, containing 2.5 m*M* heptanesulphonic acid–methanol, 42.5:57.5, v/v). At 11.5 min the concentrating column was switched off and eluted with mobile phase B (methanol containing 2.5 m*M* heptanesulphonic acid). The total time of analysis was 20 min.

HPLC with electrochemical detection

The HPLC equipment consisted of the following components: a manual injector (Rheodyne Model 7125) equipped with a sample loop of 20 μl, a solvent delivery system (Waters Model 590), an electrochemical detector (Antec, Leiden, The Netherlands) operating at 0.8 V and an recorder (Kipp en Zonen BD-41, Delft, The Netherlands). The column (150 × 4.6 mm I.D.) with 2-μm frits and Valco fittings (Chrompack, Middelburg, The Netherlands) was packed with Hypersil ODS (particle size 5 μm, Shandon, Zeist, The Netherlands) using a column-packing instrument (Shandon) according to the manufacturers instructions. The following HPLC conditions were used: the column was eluted isocratically with a mixture of phosphate buffer (0.05 *M*, pH 2.5, containing 2.5 m*M* heptanesulphonic acid and 60 mg/l sodium EDTA)–methanol (40:60, v/v). The flow-rate was 1 ml/min at room temperature.

Derivatization with FMOC-Cl

To a 100-μl sample or standard were added 100 μl of borate buffer (0.5 *M*, pH

7.7) and 100 μl of a solution of FMOC-Cl (2.5 m*M* in dry acetone). After a reaction time of 45 s at room temperature, 200 μl of a solution of 1-adamantylamine (12 m*M* in acetonitrile) was added to stop the derivatization by reaction with excess of FMOC-Cl.

HPLC separation of FMOC derivatives

The HPLC equipment consisted of the following components: an automatic injector (Model WISP, Waters, Etten Leur, The Netherlands), two solvent delivery systems (LBK Model 2150) controlled by a gradient controller (LKB Model 2152), a UV–VIS detector (Linear Model 200), a data acquisition system (Axxiom, Analytica). The column consists of a cartridge of Chromspher C_{18} (100 × 3 mm I.D., 5 μm) with a pre-column (10 × 3 mm I.D., 30 μm) (Chrompack) and was maintained at 35°C with an electric column over (LKB Model 2155). Detection was performed with a UV–VIS detector at 260 nm (Linear Model 200, Analytica) or with fluorescence with excitation at 260 nm and emission at 320 nm (Shimadzu Model RF530, Lamers and Pleuger, 's Hertogenbosch, The Netherlands).

The following HPLC conditions were used: from 0 to 40 min a linear gradient from 100% mobile phase A (a mixture of sodium acetate buffer, 0.05 *M*, pH 4.2–methanol, 60:40, v/v) to 100% mobile phase B (a mixture of sodium acetate buffer, 0.05 *M*, pH 4.2–methanol–acetonitrile, 20:60:20, v/v/v), from 40 to 45 min 100% mobile phase B. The flow-rate was 0.7 ml/min.

Derivatization with dabsyl chloride

To a 20-μl sample or standard were added 40 μl of carbonate buffer (0.05 *M*, pH 8.5) and 40 μl of a solution of dabsyl chloride (4 m*M* in acetonitrile). After a reaction time of 15 min at 70°C, 100 μl of a sodium acetate buffer (25 m*M*, pH 6.5) were added to stop the derivatization.

HPLC separation of dabsyl derivatives

The HPLC equipment used for separation and detection of dabsyl derivatives is similar to that described for the separation of FMOC derivatives. The wavelength of the UV–VIS detector was set to 436 nm.

The following HPLC conditions were used: from 0 to 15 min a linear gradient from 100% mobile phase A (a mixture of sodium acetate buffer, 0.025 *M*, pH 6.5–methanol, 30:70, v/v) to 100% mobile phase B (a mixture of sodium acetate buffer, 0.025 *M*, pH 6.5–methanol, 10:90, v/v), from 15 to 20 min 100% mobile phase B. The flow-rate was 0.7 ml/min.

RESULTS

Four different detection methods for thyrosine and thyronine residues will be compared with respect to HPLC separation, time of analysis, limit of detection and practical applicability.

UV detection of parent compounds

The thyroid hormones and precursors can be detected directly at a wavelength of 230 nm. For the separation of all seven compounds, a reversed-phase HPLC

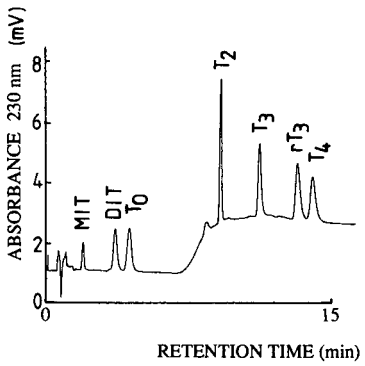

Fig. 1. HPLC profile of a standard mixture of tyrosines and thyronines as detected directly at 230 nm. A cartridge of Chromspher C_{18} (100 × 3 mm I.D., 5 μm) was used and eluted for 3.5 min with a mixture of phosphate buffer, 0.05 *M*, pH 2.5, containing 2.5 m*M* heptanesulphonic acid–methanol (65:35, v/v) and for 14.5 min with a mixture of phosphate buffer, 0.05 *M*, pH 2.5, containing 2.5 m*M* heptanesulphonic acid–methanol (42.5:57.5, v/v).

system has been developed with a stepwise gradient. In addition a pre-concentration column is used with column switching in order to avoid the appearence of matrix components of the analytical column. In Fig. 1 the chromatogram of the separation of all seven components is shown. The limits of detection are in the range 310–900 pg per injection. The individual data are listed in Table I.

Electrochemical detection of parent compounds

In general, phenolic compounds are suitable for electrochemical detection. This sensitive detection method has been applied here on four thyroid hormones, T2, T3, rT3 and T4, as shown in Fig. 2. Limits of detection have been determined at 800 mV as 0.7, 1.0, 0.2 and 0.4 pg per injection, respectively. These detection limits can probably be increased at higher voltages. For instance, at 900 mV the electrochemical responses are increased by factors of 6, 3, 2 and 1.5. Although electrochemical detec-

TABLE I

LIMIT OF DETECTION (SIGNAL-TO-NOISE RATIO = 3) FOR THYRONINE AND TYROSINE RESIDUES WITH SEVERAL DETECTION METHODS

Compound	UV (230 nm)		Dabsyl		FMOC UV		Electrochemical	
	pg	pmol	pg	pmol	pg	pmol	pg	pmol
TYR	360	2.0	80	0.44	–	–	–	–
MIT	360	1.2	120	0.39	650	2.1	–	–
DIT	900	2.1	470	1.1	800	1.9	–	–
T0	730	2.7	100	0.36	500	1.8	–	–
T2	310	0.6	190	0.36	1000	1.9	2000	3.8
T3	590	0.9	230	0.35	2300	3.5	3000	4.6
rT3	840	1.3	330	0.51	1300	2.0	250	0.38
T4	960	1.2	300	0.39	1200	1.6	1000	1.3

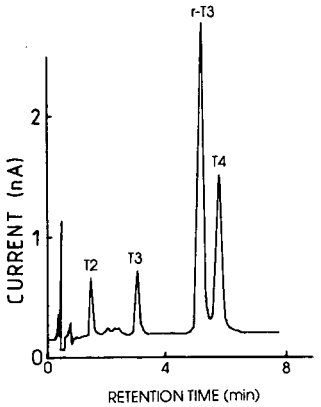

Fig. 2. HPLC profile of a standard mixture of thyroid hormones as determined with electrochemical detection. A cartridge of Chromphere C_{18} (100 × 3 mm I.D., 5 μm) was used and eluted isocratically with a mixture of a phosphate buffer (0.05 *M*, pH 2.5, containing 2.5 m*M* heptanesulphonic acid and 60 mg/l sodium EDTA)–methanol (40:60, v/v).

tion using the new Antec detector was intended as a ready-to-use and practical method of detection, this technique seems to be less suitable for automatic unattended HPLC analysis.

Fluorescence and UV detection of FMOC derivatives

Firstly, the derivatization yield was determined as function of pH. Between pH 6.5 and 9.0 no significant changes in the formation of FMOC derivatives were found. Derivatization with FMOC-Cl results mainly in mono-substituted derivatives. To a lesser extent di-substituted derivatives are formed, which can be observed in the HPLC profile at higher retention times. This may interfere with correct quantitation of the compounds. The FMOC derivatives of thyroid hormones and precursors can be detected by UV at 260 nm, with fluorescence with excitation at 260 nm and emission at 320 nm. In Fig. 3 the two detection techniques have been compared. In our hands UV detection gave the best results with respect to background signals. The fluorescence may be more sensitive but in the chromatogram high peaks also appeared in blank samples without any amino acid. This phenomenon can probably be ascribed to the preparation of FMOC-Cl which was only 95% pure. Limits of detection with UV detection ranged from 0.5 to 2.3 ng per injection. The data for the individual compounds are listed in Table I. The limits of detection with fluorescence could not be determined, as mentioned before.

Detection of dabsyl derivatives

The optimization of the derivatization with dabsyl chloride has been described in detail elsewhere [10]. The dabsyl conjugates of the thyroid hormones are all di-substituted derivatives. Besides the amino group, the phenolic hydroxyl group is also susceptible to conjugation. As a result the dabsyl conjugates elute at a much higher methanol concentration from the C_{18} column than the other naturally occurring amino acids. In Fig. 4 a separation of eight tyrosine and thyronine derivatives is

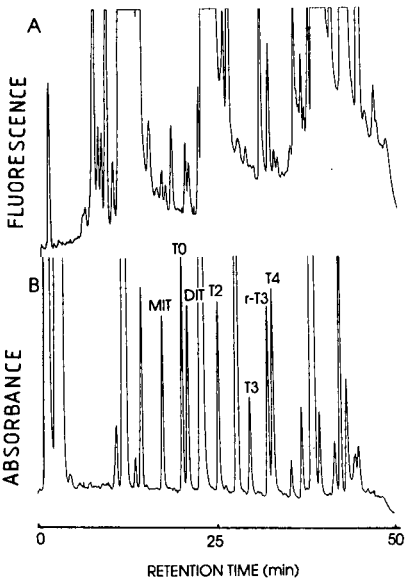

Fig. 3. HPLC profiles of a standard mixture of FMOC derivatives of tyrosines and thyronines as detected with fluorescence (A) and UV (B). A cartridge of Chromspher C_{18} (100 × 3 mm I.D., 5 μm) was used and eluted from 0 to 40 min with a linear gradient from a mixture of sodium acetate buffer, 0.05 *M*, pH 4.2–methanol (60:40, v/v) to a mixture of sodium acetate buffer, 0.05 *M*, pH 4.2–methanol–acetonitrile (20:60:20, v/v/v).

shown with a complete baseline separation. The limits of detection are very good, ranging from 80 to 470 pg injected on the column with a signal-to-noise ratio of 3. The individual detection limits are listed in Table I.

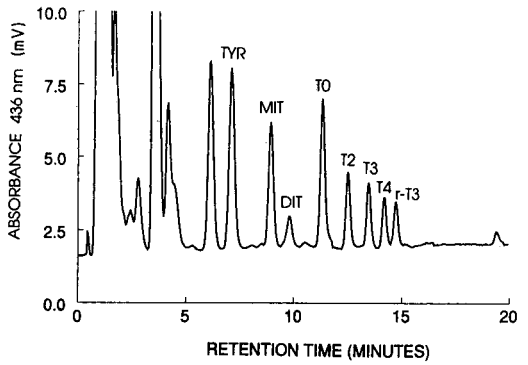

Fig. 4. HPLC profile of a standard mixture of dabsyl derivatives of tyrosines and thyronines as detected at 436 nm. A cartridge of Chromspher C_{18} (100 × 3 mm I.D., 5 μm) was used and eluted from 0 to 15 min a linear gradient from a mixture of sodium acetate buffer, 0.025 *M*, pH 6.5–methanol (30:70, v/v) to a mixture of sodium acetate buffer, 0.025 *M*, pH 6.5–methanol (10:90, v/v).

Samples from toxicological experiments

The HPLC method with UV detection at 230 nm was selected as the most practical method of analysis. With this method a number of toxicological experiments on the thyroid have been performed. Thyroid precursors and hormones have been determined in the thyroidal protein called thyroglobulin, which consists to a large extent of tyrosine residues. In this protein the synthesis of the precursors MIT and DIT takes place by iodination of tyrosine, and the synthesis of the hormones T3, rT3 and T4 by the coupling of the various thyrosines to thyronines by thyroid peroxidase. A method was developed for total proteolytic hydrolysis of thyroglobulin [11] followed by HPLC analysis of the residue to determine the concentrations of the precursors and the thyroid hormones. In Fig. 5 an example of a typical chromatogram is shown from the hydrolysate of the thyroid from a rat which has been treated with sodium bromide. Experimental conditions and detailed data of analysis will be published elsewhere.

DISCUSSION

The detection of thyroid hormones and precursors such as TYR, MIT, DIT, T0, T2, T3, rT3 and T4 was investigated with four different HPLC methods. For all methods a satisfactory baseline separation can be shown. The parent compounds were investigated with two methods: UV detection and electrochemical detection. The UV detection of the parent compounds showed rather low limits of detection (from 310 to 960 pg per injection) caused by the high intrinsic extinction coefficients

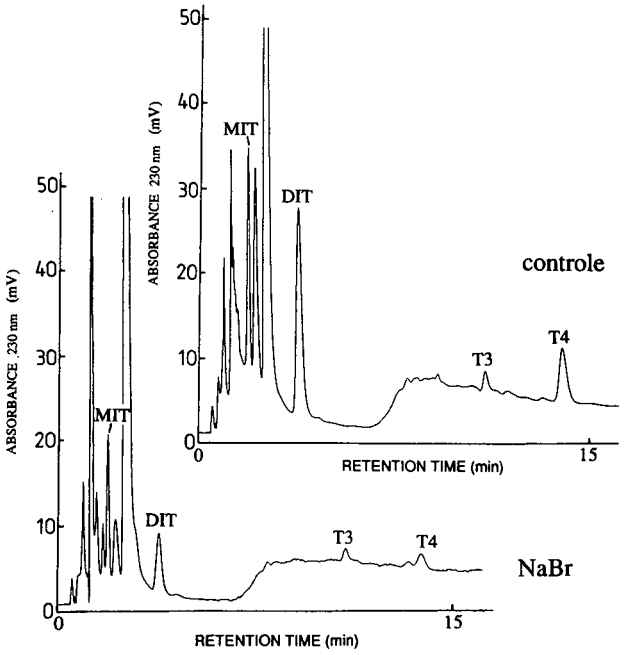

Fig. 5. HPLC profiles of the detection of thyroid tissue from a control rat and a rat treated with sodium bromide. Experimental details have been described in the Experimental section.

of the parent compounds. The detection limits for the individual compounds are listed in Table I. Electrochemical detection was a practical technique without many problems. This could probably be ascribed to the new Antec detector. The limit of detection ranged from 0.25 to 3 ng per injection and can probably be decreased by measurements at higher oxidation potentials. However, the UV detection was judged to be of more practical value.

To increase the sensitivity, two pre-column derivatization methods were investigated: dabsyl and FMOC. These were chosen because, as already described for amino acids, they yield stable derivatives. Both the FMOC and the dabsyl derivatives are stable at least for several hours or even days. In addition, both derivatives can be detected with great sensitivity. The FMOC derivatives can even be detected with fluorescence. In our experience the detection with fluorescence was not satisfactory because too many high peaks originating from the reagent blank appeared in the chromatogram. A similar observation was made in our laboratory with the derivatization of aminoglycosides with FMOC-Cl. The limit of detection of the dabsyl derivatives differed by a factor 2–10 compared with the FMOC derivatives with UV detection. In addition, the detection of the dabsyl derivatives take place at 436 or 450 nm where no disturbance peaks from matrix components are expected. For these reasons the dabsyl derivatization was preferred.

The direct detection of the parent compounds at 230 nm showed somewhat higher limits of detection (by a factor 1.5 to 7) than the dabsyl derivatives. However, for good quantitation the extra steps necessary to perform a derivatization can only decrease the reliability. In addition, a number of parameters involved in the derivatization must be investigated thoroughly, such as extent of derivatization, the influence of matrix components, the sustainability of the method with respect to pH, type of buffer, ionic strength, temperature, time of derivatization, etc. Therefore we have chosen UV detection of the parent compounds as the most practical method, with dabsyl derivatization as second best to achieve a higher sensitivity if necessary.

With UV detection a number of samples from a toxicological experiment with sodium bromide were analyzed and it was concluded that the selected method described here has also proven very suitable in practice.

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CHROMSYMP. 2342

Normal- and reversed-phase high-performance liquid chromatography of some phosphonodipeptides

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ABSTRACT

The chromatographic behaviour of some phosphonodipeptides on octadecyl, nitrile and aminopropyl sorbents was studied. The retention and selectivity parameters of the separation of phosphonopeptide diastereoisomers on the sorbents were determined. The diastereoisomers of the unprotected phosphonopeptides can be separated at pH <5 by reversed-phase high-performance liquid chroamtography. The separation of the protected diastereoisomers can be obtained on nitrile and aminopropyl sorbents by use hexane–isopropanol eluents.

INTRODUCTION

Aminophosphonic acids and peptides have high biological activity [1,2]. It is important to obtain these compounds with high optical purity, but few studies have been devoted to the liquid chromatography of phosphonopeptides. The separation of diethyl esters of 1-(N-L-alanylamino)benzylphosphonic acid isomers by ion-exchange chromatography has been described [3]. The diastereoisomers of L-phenylalanyl-1-aminoalkylphosphonic acids dialkyl esters have been separated on silica, but not very succesfully [4].

The aim of this work was to study the possibilities of applying different modes of high-performance liquid chromatography (HPLC) for the separation of phosphonopeptide (PP) diastereoisomers that differ in the configuration of the α-carbon atom of the aminophosphonic acid residues.

EXPERIMENTAL

Chromatographic conditions

The experiments were performed on an LKB (Bromma, Sweden) liquid chromatographic system consisting of a Model 2150 HPLC pump, a Model 7410 injector, a Model 2140 rapid spectral detector at 225 nm and a Model 2200 recording integrator. The column used were as follows: (1) octadecyl polyol Si 100, 5 μm, 250 × 4.6 mm I.D., (Serva, Heidelberg, Germany); (2) Separon SIX C_{18}; (3) Separon SIX NH_2; and (4) Separon SIX CN (the last three all 5 μm, 150 × 3.3 mm I.D.) (Tessek,

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Prague, Czechoslovakia). The mobile phases were methanol–10^{-2} *M* phosphate buffer (0:100 to 60:40) (10^{-2} *M* H_3PO_4 adjusted with ammonia) for columns 1 and 2 and hexane–isopropanol (1:99–20:80) for columns 3 and 4. The flow-rate was 0.5 ml/min for column 1 and 0.25 ml/min for columns 2–4.

Materials

Phosphonopeptides were obtained as described [5,6]. Methanol, hexane, isopropanol, orthophosphoric acid and ammonia (analytical-reagent grade) were used as received. Water was doubly distilled and filtered for HPLC use.

RESULTS AND DISCUSSION

The least hydrophobic phosphonopeptide L-Ala-L,D-Ala P[a] (I) has a low retention on octadecyl sorbent. In this instance it is necessary to use eluents with high ionic strength. The retention and selectivity (α) of the separation of PP diastereomers increase with increasing concentration of ammonium sulphate in the mobile phase (MP) (Table I). For L-Val–L,D-Ala P (II) the selectivity of the separation of the diastereoisomers is 2.16 when the MP does not include methanol.

TABLE I

RETENTION OF L–D ISOMERS (k') AND SELECTIVITY OF SEPARATION OF PP DIASTEREOISOMERS

Peptide	Column	Methanol (%)	k'_2	$\alpha = k'_2/k'_1$
I	1	1.5[a]	1.469	2.17
		3.0[a]	3.384	5.76
II	1	0	0.735	2.16
		10	0.337	1.55
III	1	30	2.145	1.4
		40	1.02	1.43
	2	30	9.11	1.73
		40	4.72	1.65
VI	1	62	4.26	1.17

[a] Concentration of $(NH_4)_2SO_4$ (*M*).

A further increase in the hydrophobicity of dipeptides [L-Leu–D,L-Phe P (III) and L-Phe–L,D-Leu P (IV)] does not result in an improved separation of diastereoisomers. A comparison of the chromatographic behaviour of dipeptide L-Leu–D,L-Phe (V) with its phosphonic analogues (III) shows that the latter is characterized, under comparable conditions, by both a smaller retention time and a smaller selectivity of separation of diastereoisomers (Fig. 1). Replacement of a carboxylic by a phosphonic group has a stronger effect on the retention of L,D-peptides.

[a] Abbreviations: we generally used accepted abbreviations for α-aminophosphonic acids with index P, *e.g.*, L,D-1-(N-L-alanylamino)ethylphosphonic acids (L-Ala–L,D-Ala P), diethyl ester of L,D-1-(N-benzyloxycarbonyl-L-phenylalanylamino)-3-methylbutylphosphonic acid [*Z*-L-Phe–L,D-Leu $P(OEt)_2$].

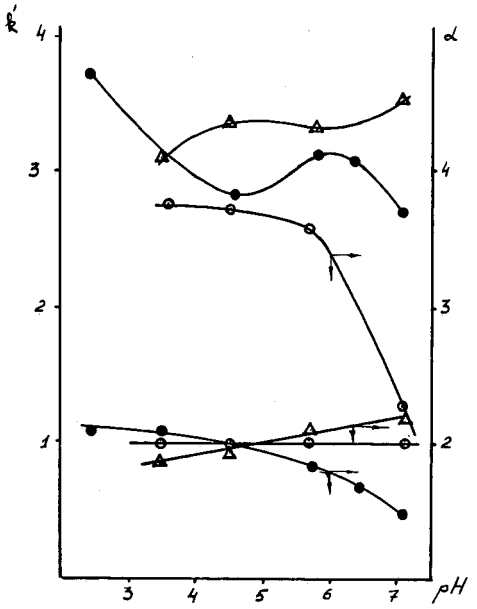

Fig. 1. Influence of pH on capacity factors (k') of L–L isomers and selectivity (α) of PP diastereoisomer separation by RP-HPLC. Compounds: ○ = II; ● = III; △ = V. For conditions, see Experimental.

The retention time and selectivity of separation of phosphonodipeptide diastereoisomers increase appreciably with decreasing pH in the range 5–7, which corresponds to one of the pK values for dissociation of a phosphonic group (p$K \approx$ 5–6) (Fig. 1). The amino acid sequence of isomeric PP [L-Leu–L,D-Phe P (III) and L-Phe–L,D-Leu P (IV) has a weak effect on the retention time of L,L-diastereoisomers, whereas the retention of L,D-diastereoisomers is affected more appreciably, resulting in different selectivities (Fig. 2).

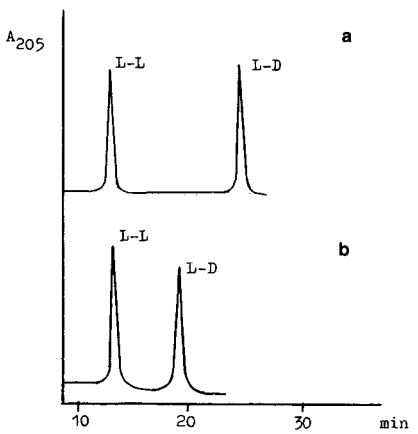

Fig. 2. Chromatograms of the separation of PP diastereoisomers by RP-HPLC. Column 1; eluent, methanol–0.01 M phosphate buffer (pH = 7.1) (40:60); flow-rate 0.5 ml/min. Compounds: (a) IV and (b) (III).

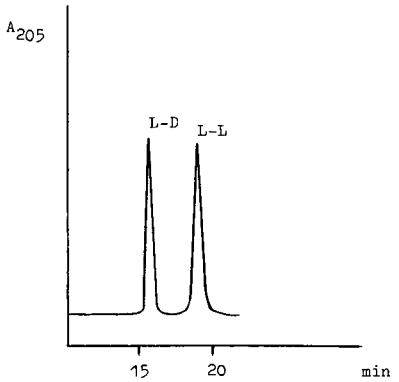

Fig. 3. Chromatogram of the separation of PP diastereoisomers (IX). Column 4: eluent, hexane–isopropanol (98:2); flow-rate, 1.0 ml/min.

Protected peptides are sorbed much more efficiently on octadecyl sorbents, but it is possible to separate diastereoisomers on these only for the (*Z*)-L-Phe–D,L-Leu $P(OEt_2)_2$ (VI). Clearly, in protected PP with a sorbent the interaction occurs primarily between hydrophobic protected groups, whereas in unprotected compounds the dominant contribution to the interaction comes from those parts of the molecules which differ in their configurations.

To eliminate or decrease the contribution from hydrophobic protected groups to the binding with a sorbent, we applied chromatography on nitrile and aminopropyl sorbents with hexane–isopropanol mixtures as MP to separate protected PP (Fig. 3).

Table II gives the parameters of the equation that describes the dependence of the retention of isomers on the concentration of isopropanol in the MP. We succeeded in separating the diastereoisomers of all the protected PP studied.

Under identical conditions [hexane–isopropnol (99:1)], the selectivity of sep-

TABLE II

COEFFICIENTS OF THE EQUATION $\ln k' = A - B\ln C$

Eluent: isopropanol [concentration C% (v/v), range 1–20%] in hexane.

Peptide	Column	L–D Isomers		L–L Isomers	
		A	*B*	*A*	*B*
VI	3	3.21	1.25	3.31	1.29
	4	2.88	1.44	2.88	1.44
VII	3	3.01	1.29	3.55	1.41
	4	2.16	1.31	2.76	1.50
VIII	3	2.11	1.11	2.43	1.19
	4	1.38	1.27	1.56	1.34
IX	3	3.63	1.30	3.92	1.40
	4	3.28	1.44	3.48	1.52

aration of diastereoisomers on the nitrile sorbent decreases in the order (*Z*)-L-Val–L,D-Ala P(OiPr)$_2$ (VII) > Boc-L-Leu–L,D-Phe P (OiPr)$_2$ (VIII) > (*Z*)-Ala–L,D-Ala P(OiPr)$_2$ (IX), and on the aminopropyl sorbent in the order VII > VIII > VI. Generally, the selectivity of separation and retention of protected phosphonic dipeptides is higher on the nitrile sorbent

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CHROMSYMP. 2135

Rapid and sensitive high-performance liquid chromatographic method for the analysis of tryptophan, tyrosine and phenylalanine in biological samples

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ABSTRACT

In order to overcome problems related to the determination of free tryptophan in biological fluids using conventional methods, we have developed an accurate and reliable procedure based on a specific pretreatment of samples followed by a very rapid and sensitive reversed-phase high-performance liquid chromatographic analysis. The pretreatment consists of adding to the sample of a very low amount of 3 *M* phosphate buffer to maintain pH in the physiological range followed by ultrafiltration. The precision, reproducibility and sensitivity of our method were also evaluated. The recovery of each amino acid was greater than 92%. The use of a microbore column allows the detection of up to 0.2 pmol/μl of amino acid. The method has been applied to the analysis of samples obtained from 25 normal and 10 phenylketonuric subjects.

INTRODUCTION

Tryoptophan is the only amino acid in blood which is bound to a large extent to albumin. Several studies have demonstrated that the degree of binding of Trp to serum albumin is an important factor in the unidirectional uptake of Trp into the brain [1–6]. The binding of Trp to albumin is of non-covalent type and is strongly influenced by blood composition (by its content of fatty acids, drugs and different macronutrients), ionic strength and pH [1,7-10]. For this reason, the usual methods of assessing total Trp content by amino acid analysis do not provide reliable information about the blood level of free Trp [11]. Specific techniques which have been used for this purpose, such as equilibrium dialysis and ultrafiltration, do not meet the requirements of a rapid and sensitive analysis [12,13].

The aim of this study was to establish a rapid and reliable method to determine

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the concentration of plasma free Trp using a new pretreatment procedure followed by a rapid and sensitive high-performance liquid chromatographic (HPLC) analysis with UV detection of tryptophan, tyrosine and phenylalanine.

EXPERIMENTAL AND METHODS

L-Amino acid crystalline salts and other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) and HPLC-grade solvents from Behring (Riedel-de Haen). The Kontron (Zurich, Switzerland) HPLC system consisted of two HPLC pumps, a 460 autosampler and a 480 column oven, all under computer control; the detection system was a Waters (Millipore, Bedford, MA, U.S.A.) 990 photodiode array detector.

Freshly drawn human blood samples were collected in light-shielded, heparinized vacuum containers to prevent photo-oxidation of Trp and centrifuged at 1085 *g* for 15 min. To prevent pH changes, 10 μl of 3 *M* sodium monohydrogenphosphate (Na_2HPO_4), pH 6.6, were added to 400 μl of plasma to obtain a plasma pH of 7.4 $\pm$ 0.01.

A 200-μl sample of plasma was immediately centrifuged at 27 000 *g* for 20 min with Ultrafree M.C. 10 000 NMWL (Millipore), and 20 μl were then injected onto a Spherisorb ODSl column (5 μm, 25 cm $\times$ 4.6 mm I.D.) (Phase Separation, Waddinxveen, The Netherlands). The column was maintained at a constant temperature of 40°C. The elution was performed at a flow-rate of 1.1 ml/min using a gradient mobile phase composed of 12 m*M* phosphate buffer (pH 2.8) and acetonitrile–12 m*M* phosphate buffer (pH 2.8) (50:50, v/v). The eluent composition profile is shown in Fig. 1. For the sensitivity assay we also used a Spherisorb ODS2 microbore column (3 μm, 15 cm $\times$ 2 mm I.D.) at a flow-rate of 0.2 μl/min.

The measurement of total Trp was performed by adding 100 μl of plasma to 400 μl of 10% trichloroacetic acid (TCA). The sample was then centrifuged at 14 400 *g* for 15 min. A 100-μl sample of the supernatant was mixed with 100 μl of 0.3 *M* phosphate buffer (pH 7.3) in order to achieve a pH of 2.5 before injecting 20 μl into the chromatographic system.

Tyr, Phe and Trp were quantified using UV absorption at 220 nm.

RESULTS AND DISCUSSION

Changes in the plasma free Trp concentration influence the brain Trp concentration and hence brain turnover of 5-hydroxytryptamine [2,5,9]. Furthermore, a circadian rhythm of Trp plasma values has been reported by several authors [3,9,14]. It is therefore important, in order to perform clinical studies, that there should be available a fast, sensitive and precise method of assaying free and total Trp. The simultaneous determination of Tyr and Phe gives further information on the plasma concentration of neurotransmitter precursors. The method developed in our laboratory allows a good separation of Tyr, Phe and Trp from other UV-absorbing plasma constituents (Figs. 1 and 2).

The dose–response relationship was found to be linear over the ranges 2–100 μM for Tyr and Trp and 10–1000 μM for Phe, and in each case the correlation coefficient was greater than 0.9993.

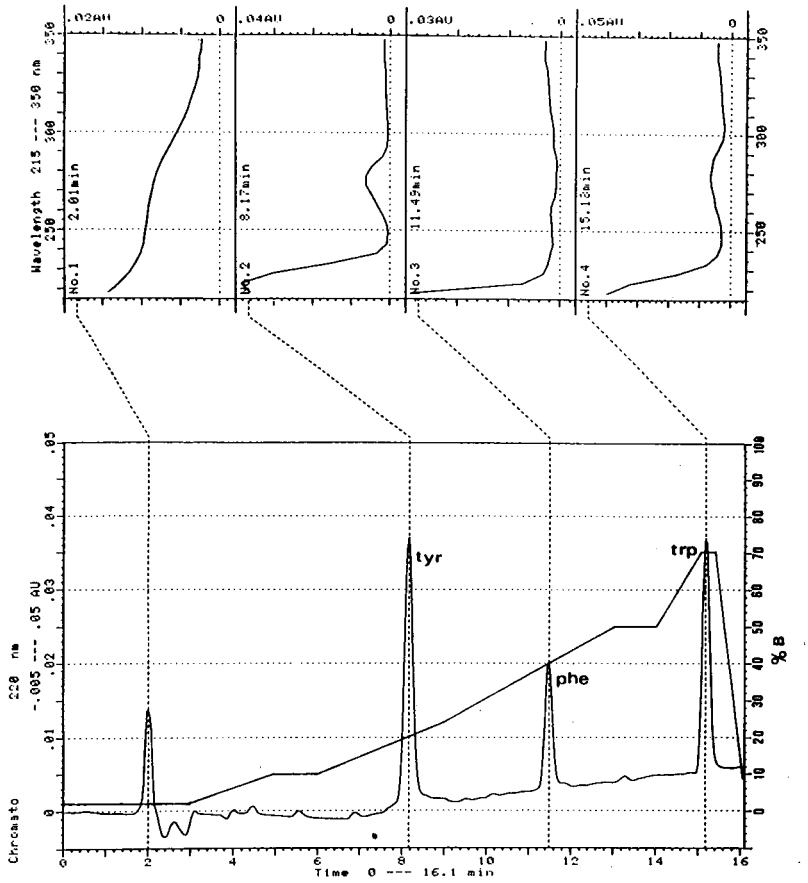

Fig. 1. Chromatogram and spectra of a standard solution containing Tyr (50 μM), Phe (100 μM) and Trp (10 μM).

Using a conventional column, the minimum measurable amount of each amino acid was 2 pmol/μl; by using a microbore column the minimum measurable amount of each amino acid was 0.2 pmol/μl.

In most studies of protein-bound analytes, the stability of pH conditions during sample treatment is critical. This is particularly true in our case; we therefore evaluated the free Trp to total Trp ratio in plasma at different pH and dilutions. Our studies confirmed that there is a significant decrease in free Trp with increase in pH (Table I) and therefore it is necessary to maintain the pH stable within the physiological range during sample treatment. In agreement with Eccleston [12], we also found that increasing the dilution of the plasma with phosphate buffer, under the same conditions of pH and molarity, results in an increase of the free Trp fraction (Table II). These observations indicate that the optimum pH is 7.4 $\pm$ 0.02 at the minimum dilution of the plasma with phosphate buffer (1:1.025).

The method was tested using 25 reference samples taken from healthy consent-

TABLE I

EFFECT OF PLASMA pH CHANGES ON FREE Trp VALUE

Free Trp level variations at different pH were tested by adding 0.3 *M* phosphate buffer, at various pH, to plasma in a 1:1 ratio. Mean values ± S.D. were calculated from five replicated samples.

Sample pH	Free Trp (μM)	Bound Trp (μM)	Free Trp (%)
6.7	31.8 ± 0.2	32.5 ± 0.3	49
7.0	29.5 ± 0.4	34.8 ± 0.2	46
7.3	19.9 ± 0.1	44.4 ± 0.2	31
7.4	17.8 ± 0.3	46.5 ± 0.4	28
7.7	12.6 ± 0.3	51.7 ± 0.6	19
8.5	6.8 ± 0.2	57.5 ± 0.5	10

ing adults after an overnight fast. Plasma total Trp values were 50.4 ± 11 μM; free Trp values were 10.1 ± 5 μM, and the mean free Trp percentage was 20.7 ± 3. Phe mean values were 61 ± 7 μM, and Tyr mean values were 63 ± 8μM. The method was then applied to the analysis of samples obtained from ten hyperphenylalaninemic subjects (Fig. 2).

The values found were in the same range as reported by previous studies [11], except for free Trp. In our case the values obtained were slightly higher than those reported by Eccleston [12] and Flentge *et al.* [13]. Despite the small number of reference samples so far tested, we believe that the small variability observed is satisfactory.

Recovery studies for total Trp were performed by adding different amounts of the amino acid to a blood sample containing a known concentration of Trp. The mean recovery was 97%. This agrees well with other reports. Recoveries of Tyr and Phe were evaluated in a similar manner. The mean recoveries were 102 ± 8% for Tyr and 92 ± 4% for Phe.

Ten samples with known concentrations of Tyr and Phe were analysed by our method and by an ion-exchange amino acid analyser. Good correlations were obtained with both analytes. The correlation coefficient was 0.98 for Tyr and 0.967 for Phe.

TABLE II

EFFECT OF PLASMA DILUTION ON FREE Trp VALUE

Data were obtained by diluting a plasma sample with 0.3 *M* phosphate buffer, pH 7.3. Mean values ± S.D. were calculated from five replicated samples.

% Plasma Dilution (%)	Free Trp (μM)	Bound Trp (μM)	Free Trp (%)
97	10.6 ± 0.3	42.6 ± 1	20
50	13.3 ± 0.2	38.5 ± 0.6	25.7
20	28.1 ± 0.2	23.7 ± 0.4	54.3
11	33.2 ± 0.4	18.6 ± 0.5	64
6	36.6 ± 0.1	15.2 ± 1.1	70.6

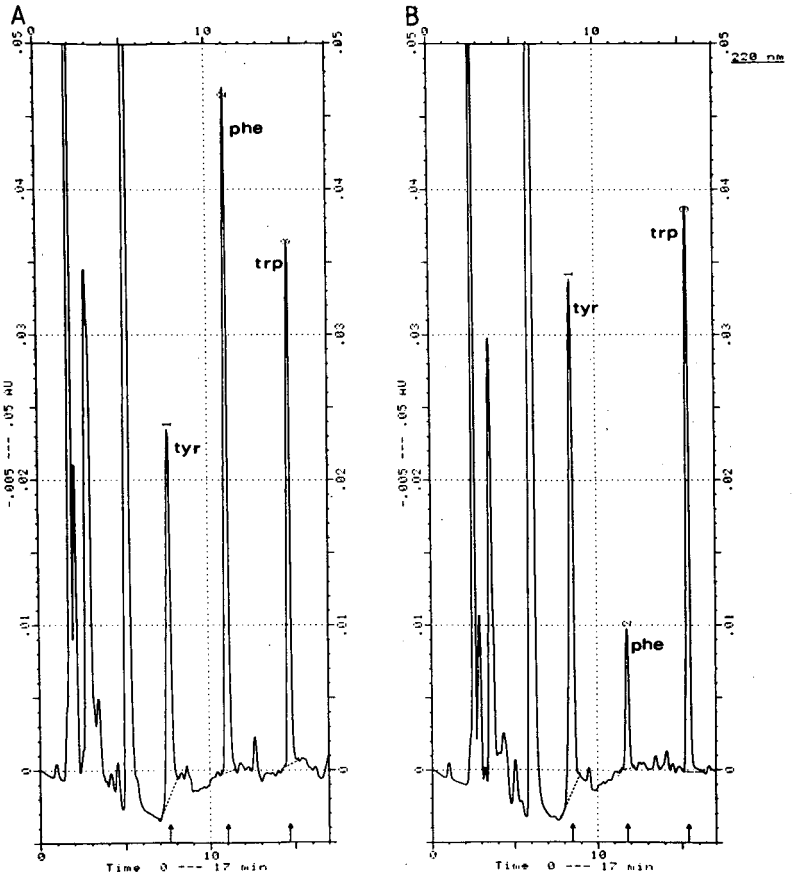

Fig. 2. Chromatograms obtained from plasma analysis of (A) a newborn affected by neonatal hyperphenylalaninaemia (Phe = 304.6 μM) and B a normal subject.

In conclusion, the low level of free Trp in plasma and the particular conditions needed for free Trp determination are strongly affected by Trp photosensitivity [15]. This sensitivity, together with the lability of albumin–tryptophan binding, has prompted us to develop new, more robust methods for Trp determination [12,13]. Most methods take advantage of the high sensitivity of fluorimetric detection, but these methods require more steps for the derivatization of the sample [15–17]. UV detection is far less sensitive but much more practical [18]. We have therefore developed a fast (15 min), precise and sensitive method of determining the concentrations of most important amino acids involved in the synthetic pathway of serotoninergic neurotransmitters.

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CHROMSYMP. 2333

Improvement of extraction and concentration of milk peptides with solid-phase cartridges for analysis by high-performance liquid chromatography

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ABSTRACT

Peptides were measured in skim milk by reversed-phase high-performance liquid chromatography after a solid-phase extraction step. Milk proteins were removed by precipitation and centrifugation after the samples were adjusted to pH 4.6. The peptides were then extracted from the supernatant using solid-phase cartridges, which had been treated to reduce irreversible non-specific adsorption of peptides. Different treatments using either α-lactalbumin or milk fractions to block the binding sites of the solid extractor have been compared. The treatment increased total peptide recovery and reduced cartridge-to-cartridge variability. Because this method effectively concentrates the peptides in a sample, it is applicable to peptide mapping of industrial milk samples.

INTRODUCTION

Peptides usually occurring in or produced by proteolysis of milk have potential applications in nutrition [1,2], flavouring [3–5] and bioregulation [6–8]. This last point has been extensively reviewed by Maubois and Leonil [9]. Functional properties of these peptides may also find applications in dairy technology [10–14]. Finally, as these peptides can serve as valuable tracers of milk proteolysis, they may be useful in the determination of quality standards.

In general, in dairy applications, the usual analytical methods are unsatisfactory in attempting to make correlations between proteolysis and specific technological parameters or sensory evaluation, owing to their lack of sensitivity and to interference of milk proteins. Typically, at present, methods for the analysis of the extent of proteolysis in skim milk or coagulum are based on trichloroacetic acid (TCA) precipitation of proteins followed by derivatization of primary amines with trinitrobenzene

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sulphonic acid, and finally measurement of optical density [4]. Until recently, chromatography of milk and milk products was restricted to separation by size exclusion, principally for the determination of caseinomacropeptide [15,16]. This peptide is purportedly a good tracer of milk proteolysis resulting from contamination of milk by psychotrophic bacteria. However, using reversed-phase high-performance liquid chromatography (HPLC), it was recently shown that caseinomacropeptide is only a minor product of the proteolysis of milk due to these bacterial contaminants [17].

Although HPLC is generally convenient for peptide analysis, it is of limited use in the case of milk products, owing to the presence of micellar proteins, lipids and other interfering material. These components are often irreversibly retained by the support, resulting in irreproducible analysis and short column life-time. For this reason, most of the published data on milk peptides obtained by reversed-phase HPLC analysis concerns peptides resulting from the action of purified enzymes on purified milk proteins [18,19]. As these proteolytic models are made in homogeneous solution and for an extended time, extraction and concentration of peptides before HPLC analysis is useless.

Thus, although these models may be useful, they are insufficient to account for the complex reactions that occur in milk. The aim of this work is to optimize the application of solid-phase extraction (SPE) techniques to the analysis of peptides in milk.

Indeed, SPE [20–24] appears to be a promising technique for peptide extraction, with application to milk product. One advantage of this technique is the concentration effect of the extraction process. This is especially important for milk peptides, which are normally present in low concentrations. A limitation of the technique is caused by the properties of solid-phase cartridges based on octadecylderivatized silica, which exhibit non-specific adsorption characteristics altering the reproducibility of measurements. The optimized protocol for the analysis of milk developed in this study includes a sample deproteinization step, followed by a peptide extraction step on treated solid-phase (TSP) cartridges obtained from different procedures, which have been compared.

EXPERIMENTAL

Materials

All chemicals and biochemicals were from Carlo Erba (La Défense, Paris, France) unless otherwise indicated. Milk was obtained from Gervais-Danone (Le Plessis-Robinson). Trifluoroacetic acid HPLC grade (TFA) and α-lactalbumin were from Sigma Chimie (La Verpillière, France). SPE cartridges (type 70206) and the extraction apparatus with manifold were from J. T. Baker (Sochibo, France). Other SPE cartridges were from Waters (ref. 20805, St Quentin en Yveline, France); Supelco (ref. 57054, St. Germain en Laye, France) and Alltech (ref. 205350, Templeur, France). Skim milk powder AFD from Prolait (Niort, France) is a reference material from Institut Technique du Gruyère (Rioz, France). Reference proteolytic hydrolysate, "Peptide N3", is from Armor Proteine (Cogles, France). The C_{18} analytical column (Nucleosil 255, 25 × 0.46 cm I.D.) was from SFCC (Neuilly Plaisance, France). The water used for HPLC analysis was produced on a Milli-RO–Milli-Q system (Millipore, St. Quentin en Yvelines, France).

The water used for the analysis was purified until the extract from 100 ml passed through an SPE cartridge produced no peak on HPLC analysis with high sensitivity UV detection (220 nm, 0.01 a.u.f.s.). As previously described [25,26], good water quality is critical, because of the concentration of its impurities during extraction on SPE-cartridges

Instrumentation

The HPLC system (Waters) consisted of a Wisp Model 710 B injector, linked to two Model 510 pumps with Model 490 UV detector and a Gilson Model 811 dynamic mixer. Gradient monitoring, data acquisition and processing were achieved on a Compaq 40 micro-computer with Maxima 820 software from Waters.

Sample processing before solid phase extraction

Solid phase extraction and HPLC analysis require the preliminary removal of casein micellea. Two methods to achieve this were compared: TCA precipitation, and casein precipitation at pH 4.6.

Protein precipitation by TCA. A 100-ml volume of 24% (w/v) aqueous TCA solution were added to 100 g of skim milk, then mixed for 30 min at 4°C. The mass was adjusted to 250 g with water, and the supernatant was collected by centrifugation at 2000 *g* for 20 min at 4°C.

Protein precipitation at pH 4.6. A milk sample was prepared by a procedure similar to Aschaffenburg method [27], as follows: to 100 g of skim milk were added 100 g of water, and 10 ml of 10% (w/w) acetic acid. The solution was mixed well for 10 min, at which time 10 ml of 1 *M* sodium acetate in water were added and mixed for 15 min. The mass was adjusted to 250 g with water. The precipitate was removed by centrifugation at 2000 *g* for 20 min at 5°C.

Extraction cartridge processing before peptide extraction

In addition to the conventional conditioning procedure, commercial SPE cartridges needed a treatment to enhance the yield and reproducibility of peptide extraction. The cartridges were first conditioned, using 12 ml of methanol followed by 12 ml of solution S1 (0.1% TFA by volume in water), and then treated by one of three different conditioning solutions: 50 ml of pH 4.6 soluble fraction of skim milk (prepared as described above) (TSP 1); or 50 ml of pH 4.6 soluble fraction of a 10% (w/w) water solution of skim milk powder (TSP 2); or 50 ml of a solution of α-lactalbumin (0.48 g/l) (TSP 3).

All treatments were performed under mild vacuum (0.5 bar), at a flow-rate of 5 ml/min. The cartridges were washed with 12 ml of solution S1, followed by 2 ml of solution S2 (acetonitrile–water–TFA, 90:10:0.1, v/v/v).

Finally, the cartridges were flushed with 18 ml of methanol and air-dried using vacuum suction (0.5 bar) for 10 min.

Dried TSP cartridges can be stored in air-tight containers for at least 2 weeks at 4°C without affecting performance.

Each treatment was performed on five cartridges. The relative standard deviation (R.S.D.), which is the standard deviation expressed as a percentage of the mean, was calculated for each chromatographic peak area (the seventeen major ones).

Skim milk peptide extraction

TSP cartridges were conditioned with 12 ml methanol followed by 12 ml of solution S1 immediately before use. Subsequently, 125 ml of the supernatant were passed through the cartridges, followed by 12 ml of solution S1. The peptides were eluted with 2 ml of solution S2. The resulting 2 ml of eluate were used for HPLC analysis.

HPLC analysis

The chromatographic conditions were: column, C_{18} Nucleosil; flow-rate, 0.8 ml/min; temperature, 20°C; mobile phase A, 0.1% (v/v) TFA in water; mobile phase B 0.1% TFA in acetonitrile–water (90:10, v/v); gradient elution, 0–47% B in 60 min, then 47–100% B in 5 min; isocratic elution, 100% solution B maintained until reaching (within 10 min) 0.122 absorbance (which is pure solvent B absorbance); injection volume, 20 μl; UV detection wavelengths, 220 nm and 280 nm in parallel, zero absorbance adjusted with 100% solution A.

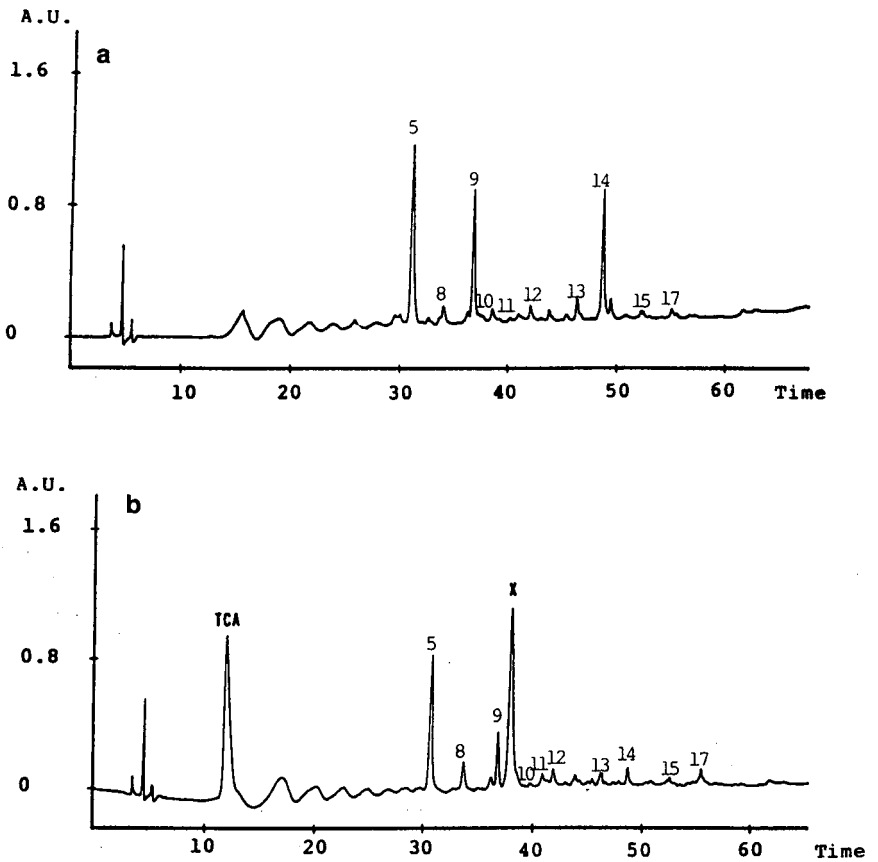

Fig. 1. HPLC analysis of peptides extracted from skim milk after various treatments for casein micellae removal. (a) Supernatant from protein precipitation at pH 4.6; chromatographic conditions as in Experimental (b) TCA supernatant (X = TCA impurity). Time in min.

RESULTS AND DISCUSSION

Milk sample pretreatment evaluation

In order to choose the optimum treatment of dairy skim milk for peptide extraction, two techniques that are common in dairy analysis and described above were compared (Fig. 1a and b). Peptide recovery using TCA (Fig. 1b) is poorer than with pH 4.6 treatment (Fig. 1a). In addition, contaminants in the TCA reagent interfere strongly in the subsequent chromatographic analysis.

The second method of casein removal based on pH 4.6 precipitation leads to the presence of hydrophobic and hydrophilic peptides (Fig. 1a).

This latter technique appears to be the better compromise as it allows the extraction and quantitation of most peptides.

Effects of SPE cartridge modification on extraction characteristics

The presence of strongly adsorbing sites in the beads of commercial cartridges leads to irreversible adsorption of several compounds contained in the milk samples. Therefore, incomplete recovery and variable yields are often observed with commercial cartridges. In order to overcome these drawbacks we have studied the effects of three different cartridge treatments by trying to saturate their binding sites as decribed in experimental. A typical chromatogram of an extract from milk processed on commercial SPE cartridges is shown in Fig. 2.

To evaluate the reproducibility of TSP 1, TSP 2, TSP 3 and commercial SPE cartridges, peptide extraction was carried out on five columns of each type. The

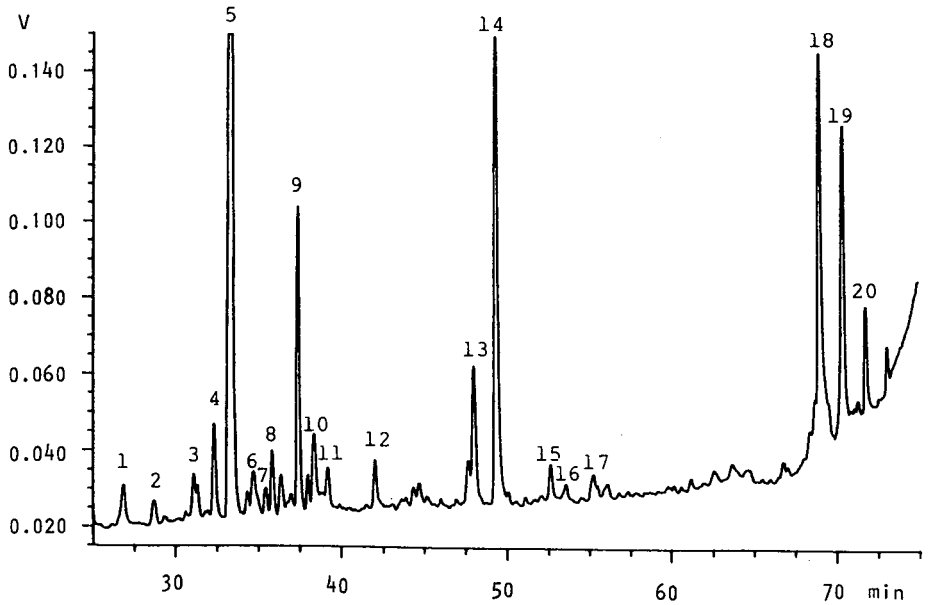

Fig. 2. Typical chromatogram of extracted peptides from dairy skim milk, after extraction on a commercial cartridge. Samples treatment and chromatographic conditions as Experimental; protein precipitation at pH 4.6, extraction of 125-ml sample on solid-phase cartridge, and injection in the HPLC system.

TABLE I

COMPARATIVE EVALUATION OF YIELD AND REPRODUCIBILITY USING TREATED (TSP) AND COMMERCIAL SPE CARTRIDGES FOR THE EXTRACTION OF MILK PEPTIDES

	Commercial	TSP 1	TSP 2	TSP 3
Lowest variation = peak 10; R.S.D. (%)	4.0	3.2	3.5	3.0
Highest variation = peak 1; R.S.D. (%)	23.2	15.0	12.8	3.8
Average yield ratio: TSP / commercial	1 (reference)	1.16	1.13	1.17

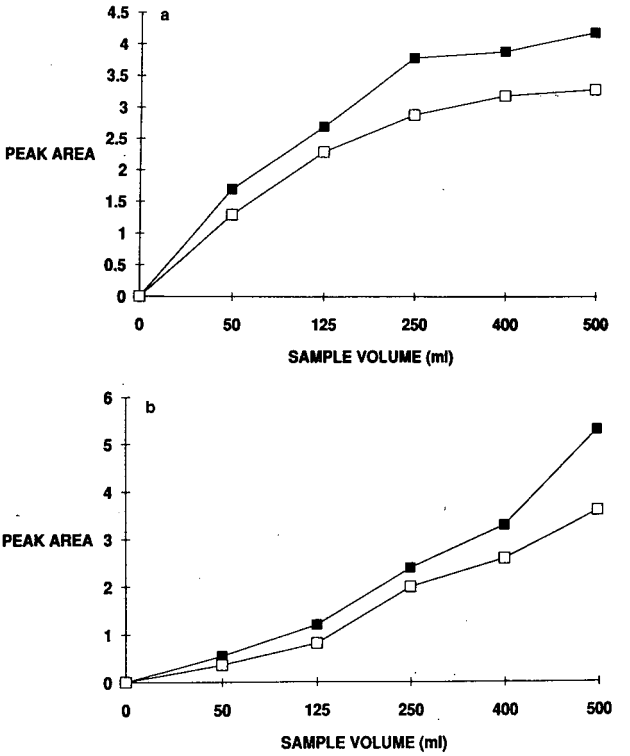

Fig. 3. Variation of HPLC peak area with extracted sample volume: Representative peaks were chosen from the chromatogram in Fig. 2. (a) Peak 13, typical of type I peptides. (b) Peak 18, typical of type II peptides. ■ = Samples extracted on TSP 3 cartridges. □ = Samples extracted on commercial cartridges. Sample treatment as in Experimental.

R.S.D. of the peak areas corresponding to the seventeen major peptides of each chromatogram were calculated as described in Experimental. The results (Table I) show that the peak areas are systematically larger and the R.S.D. smaller with the TSP cartridges, specially with TSP 3, than with commercial ones. This improvement of cartridge efficiency may be attributed to the saturation of irreversible adsorption sites (residual silanol groups) by the material present in the different conditioning solutions. In the subsequent extraction, peptide losses were minimized and cartridge capacity was standardized by using α-lactalbumin treatment (TSP 3).

Moreover, the average yield ratio calculated from the seventeen peak areas, compared with that obtained from commercial cartridges, is higher for all the treated cartridges.

The choice of α-lactalbumin (TSP 3) is based on the better control of this solution compared with the skim milk mixture. Moreover, this protein exhibits a high interaction with reversed-phase material [28,29] and its small size suggests complete access to the adsorbing sites present in the smaller pores.

In order to evaluate their effective capacities, five TSP 3 cartridges were compared with commercial ones by separately processing skim milk samples with volume 50–500 ml. The peak areas were plotted *versus* the sample volume processed in the extraction step (Fig. 3).

For all these experiments an increase in the yield of peptides extracted was observed with TSP cartridges compared with commercial SPE cartridges, and it appeared that a sample volume of 125 ml is enough for valuable analysis.

Two typical peptide retention profiles were observed. In type I, the curve plateaued after 125–250 ml of sample (Fig. 3a shows the capacity curve for peak 13), suggesting a limited number of fixation sites of the peptides considered. Type II exhibited a continuous, almost linear increase (Fig. 3b shows the capacity curve for peak 18).

This means that the difference observed between commercial and TSP cartridges is much higher than the R.S.D. and that, in all cases, TSP cartridges exhibit better performance than commercial ones. We chose peaks 13 and 18 to illustrate this effect because of their low R.S.D. deviation after five assays: peak 13 had an R.S.D. of 4.5% and 4.3% for commercial and TSP 3 cartridges, respectively, and peak 18 had an R.S.D. of 4.9% and 2.6%, respectively, measured for a volume of 125 ml.

Qualitative evaluation of the method efficiency on a milk peptide mixture

Peptide N3 (see Materials), a complex hydrolysate obtained from milk, was added to skim milk. Samples (0.16 g/l final concentration) were processed as described above with TSP cartridges (Fig. 4b). The peptide analysis was compared with direct HPLC analysis (Fig. 4a) of peptide N3 hydrolysate (4 g/l in water). Each chromatogram was run in triplicate and very similar results were obtained. Direct HPLC analysis shows at least 65 peptides of various hydrophobicities. The chromatogram from the milk extract is very similar to this reference chromatogram. Only the first five peaks, corresponding to the most hydrophilic peptides, were partially lost.

This model shows the efficiency of the method over a very large range of hydrophobicity, which allows the study of the very complex mixtures of peptides produced by the natural proteolysis of milk.

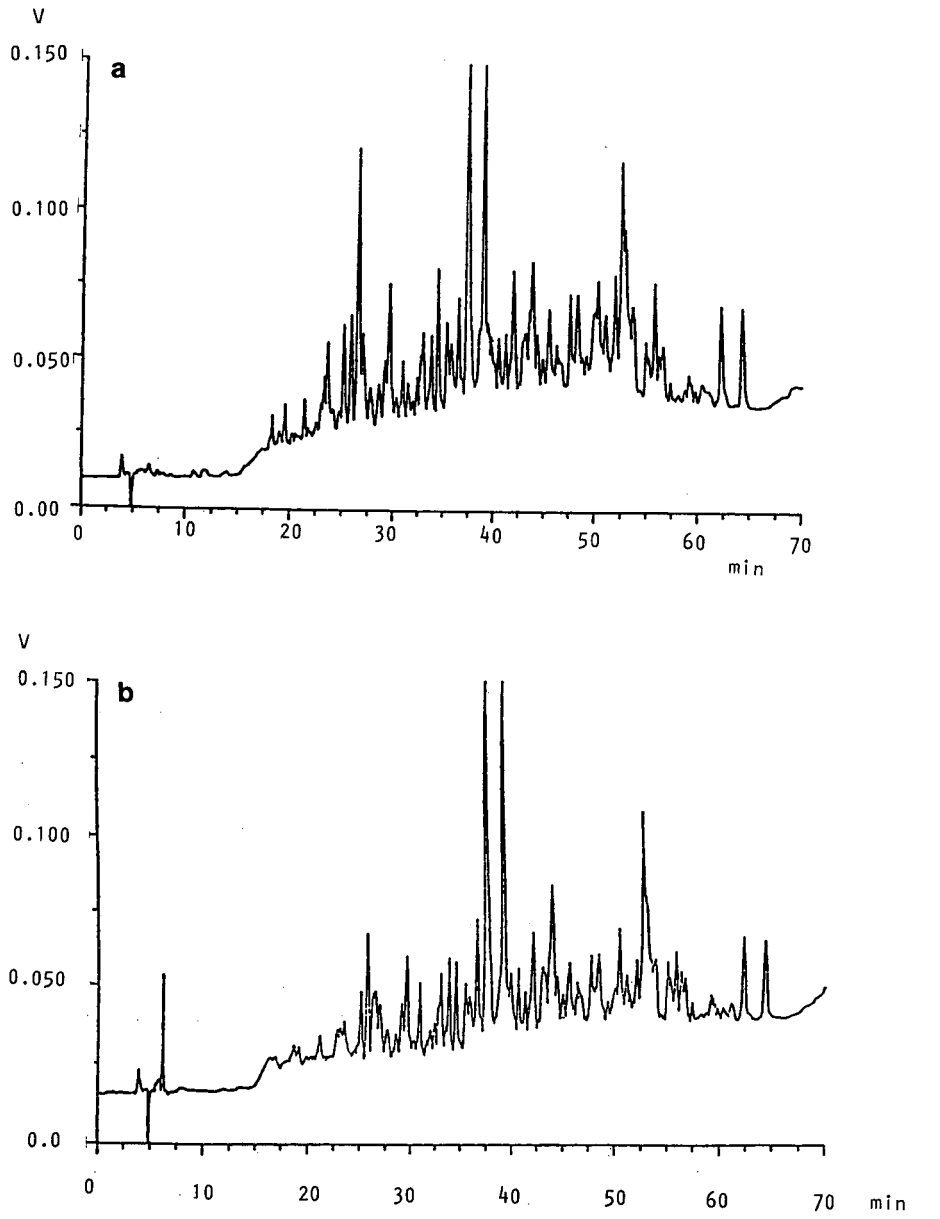

Fig. 4. HPLC analysis of peptide N3: (a) direct HPLC analysis of 4 g/l solution in water; (b) HPLC analysis of peptides extracted from peptide N3 added to skim milk at a final concentration, of 0.16 g/l.

Effects of extraction-cartridge processing on commercial SPE cartridges from different suppliers

The studies described above were all performed on commercial cartridges from J.T. Baker. The effects of treatment with α-lactalbumin (TSP 3) was also evaluated on

TABLE II

EFFECTS OF α-LACTALBUMIN TREATMENT ON CARTRIDGES FROM DIFFERENT SUPPLIERS

Supplier	R.S.D. for the total area of peaks (%)	
	Commercial cartridge	TSP 3 cartridge
J. T. Baker	11	3.6
Waters	15	6.7
Alltech	9.7	7
Supelco	11	6

cartridges from three other suppliers, Waters, Alltech and Supelco. In all the experiments the same skim milk was used. Samples were extracted on five untreated and five α-lactalbumin-treated cartridges, from each supplier. Table II sums up the results.

It appears that treatment with α-lactalbumin upgrades all four makes of cartridge, using coefficient of variation measurements and yields as comparison criteria.

CONCLUSIONS

The analytical method presented here affords a convenient way to evaluate the presence of peptides in milk and to follow the effect of proteolysis. Previously, only proteolysis by plasmin has been extensively analysed, and many products have been identified [30–41]. Some authors have reported the appearance order of peptides [41]. These data, obtained from model systems (pure proteic material solutions), do not account for the complex interactions occuring in milk or for the effective conformation of proteins in micellae.

The method described in this paper can be used to compare proteolysis occurring in real milk samples with that observed in models. It is based on HPLC evaluation of peptides in skim milk, following SPE.

The combination of protein precipitation at pH 4.6 with SPE on α-lactalbumin-treated commercial cartridges permitted the analysis of industrial milk samples with improved reproducibility. As SPE concentrates the peptides in a sample, only small volumes of milk (50 ml) are necessary. This suggests possible routine application of this method in milk quality-control laboratories.

This method has several advantages over other techniques. It gives a representative profile of the peptides in the sample, with most of the hydrophobic and hydrophilic peptides present. The reduction of non-specific adsorption on solid cartridges increases peptide recovery and significantly reduces measurement variability.

Work is currently underway to identify specific peptides that could serve as tracers of proteolysis.

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Anion-exchange chromatographic properties of α-lactalbumin eluted from quaternized polyvinylimidazole

Study of the role of the polymer coating

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ABSTRACT

The anion-exchange elution behaviour of α-lactalbumin was studied on cross-linked and quaternized polyvinylimidazole, deposited on various high-performance liquid chromatographic supports (porous silica and diol silica). The influence of the nature and thickness of the coating layer on the retention and band-width properties of the protein elution peak was examined by isocratic elution. The retention properties of α-lactalbumin were studied from the plot of log k' *vs.* log([NaCl]), where k' is the capacity factor and [NaCl] the displacer salt concentration in the aqueous phase. The retention depends on the amount of stationary phase deposited on the support, but an increased hydrophobic effect is found when the polymer films do not coat the chromatographic support uniformly. Band broadening of the elution peaks was studied in terms of plots of plate height *vs.* mobile phase velocity. An important mass-transfer contribution is found, which decreases with increasing k' and increases with the thickness of the coating layer. These effects reveal that the diffusion into the polymer layer is the controlling step of the ion-exchange process with non-uniform polymer layers of large mean thickness.

INTRODUCTION

Anion-exchange chromatography for separations of acidic proteins is a rapidly growing area, especially since the introduction of the adsorbed polymer coating technology applied to high-performance liquid chromatographic (HPLC) supports [1]. The procedure enables a broad range of selectivity to be easily obtained by varying the amount or the nature of the polymer coatings. Excellent selectivities and good protein recoveries were obtained with these stationary phases deposited on silica surfaces.

Alpert and Regnier [2] prepared supports by adsorption and cross-linking of polyethyleneimines on porous silicas. Further studies showed that the retention of proteins can be modulated by controlling the ligand density or the hydrophobicity of the polymer layer [3,4]. Adsorbed polymer technology was used by Sébille *et al.* [5] to

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develop supports for the anion-exchange chromatography of proteins by cross-linking homo- and copolymers of polyvinylimidazole (PVI) adsorbed on porous silica HPLC packings.

Good stability of the cross-linked polymer coatings was generally observed with the solvents commonly used for the HPLC of proteins. The main drawback to the use of silica as a support material is the low stability of the silica matrix at pH values higher than 8. Another limitation is the residual reactivity of the silica support towards the protein after the polymer coating. For instance, some free SiOH groups left after the polymer coating may lead to irreversible adsorption of the proteins. Other support materials were therefore selected for the ion-exchange chromatography of proteins, such as alumina [6], agarose [7], poly(methylmethacrylate) [8,9] and polystyrene–divinylbenzene copolymers [10].

Polymer coating technology involves a complex phenomenon in which various parameters may interfere, such as the nature of the support matrix and the coating procedure [11]. A better understanding of the role of these parameters is necessary for developing new ion-exchange materials based on the polymer coating technology.

In this paper we follow the work of Sébille *et al.* [5] and Boussouira [12] and describe the chromatographic properties of an anion-exchange polymeric stationary phase, quaternized polyvinylimidazole (QPVI), a PVI polymer cross-linked with epichlorohydrin. The main purpose is to understand better the role of the solid support in polymer coating technology by comparing the properties of QPVI coatings on pure porous silica and on diol silica, a matrix that can be considered as fairly inert towards proteins; the retention mechanism of proteins on diol silica is mainly size exclusion.

Although poor efficiencies are found when proteins are eluted in the isocratic mode, only a few studies have examined systematically the factors that influence the broadening of protein elution peaks [13–15]. The main reasons for the large plate heights observed in high-performance affinity chromatography are the slow adsorption–desorption kinetics [13] and the restricted diffusion of the protein into the pores [14]. Hearn *et al.* [15] have shown that the nature of the displacer salt affects the band broadening of proteins eluted isocratically from a Mono Q anion-exchange resin. On the basis of the plate-height theory, we shall examine the role of several factors that influence the performances of silica and diol silica supports coated with an anion-exchange polymer. This approach is useful for understanding better and improving the characteristics of ion-exchange polymer supports used for protein separations.

EXPERIMENTAL

Materials

The porous silica (LiChrosorb Si 100) and porous diol silica (LiChrosorb Diol) supports of particle diameter 10 μm and pore size 100 Å were purchased from Merck (Darmstad, Germany).

The reagents used for stationary phase preparation were N-vinylimidazole (NVIM) (Janssen Chimica, Beerse, Belgium), azobisisobutyronitrile (AIBN) (Eastman Kodak, Rochester, NY, U.S.A.) and epichlorohydrin (Prolabo, Paris, France).

The chemicals used for the preparation of buffers were triethanolamine and Tris (Aldrich-Chemie, Steinheim, Germany).

The protein used for the chromatographic study, calciumdepleted bovine α-lactalbumin, was obtained from Sigma (St. Louis, MO, U.S.A.).

Polymerization of PVI

The homopolymerization of NVIM has been described in detail [16–18]. We adopted the protocol of radical polymerization. PVI was synthesized by polymerization of NVIM with AIBN as a catalyst. The distilled monomer (9 g) was dissolved in 55 ml of methanol and polymerized at 60°C under a nitrogen atmosphere for 48 h with AIBN (180 mg). The polymer was precipitated in dioxane according to Palma and Chapiro [19]. The white powder obtained was collected by filtration and vacuum dried for 24 h. The reticulation and quaternization of PVI were performed in one step during the polymer coating procedure.

Polymer coating procedure

Two methods were used for the coating procedure. The adsorption method at saturation consisted in depositing the maximum amount of polymer that could be adsorbed on the supporting matrix. This method could be applied for the coating on silica, a material that can adsorb large amounts of PVI at saturation in methanol [12].

In this instance the porous support (1 g of LiChrosorb Si 100) was introduced into 10 ml of an 8% (w/v) PVI solution in methanol. It was sonicated under vacuum for 2 min in an ice-bath and then left at room temperature for 24 h. The coated support was washed with methanol and filtered in a nitrogen atmosphere and then dried. The beads were then suspended in 10 ml of methanol containing 1 ml of epichlorohydrin. The mixture was heated at 60°C for 2 h with agitation, then filtered in a nitrogen atmosphere, washed with methanol and dried at 60°C.

The other method used consists in coating the required amount of polymer on the support by complete evaporation of the solvent. This was achieved by introducing the porous support (1 g of LiChrosorb Si 100 or LiChrosorb Diol) in a solution containing the given amount of PVI. After adding 1 ml of epichlorohydrin to the mixture, sonication was applied as above. After leaving the mixture at room temperature for 24 h, the mixture was heated at 60°C for 2 h with agitation to complete the reticulation step. The solvent was then evaporated at this temperature. The coated support was washed with methanol and dried at 60°C. This method has the advantage of better control of the amount of stationary phase deposited on the support.

TABLE I

CHARACTERISTICS OF THE CHROMATOGRAPHIC SUPPORTS COATED WITH QUATERNIZED POLYVINYLIMIDAZOLE

Support	QPVI (%)	Specific surface area (m^2/g)	Exchange capacity (μequiv./g)
Silica	0	280	–
	7.6[a]	200	200
	3.8[b]	235	50
	8.5[b]	190	230
Diol silica	0	245	–
	3.0[b]	200	85
	9.0[b]	165	250

[a] Adsorbed from solution.

[b] Coated by solvent evaporation.

The exact amount of polymer on the support was determined by nitrogen elemental analysis. The characteristics of the supports studied are summarized in Table I. The particles were examined with × 100 magnification using an optical microscope (BHB, Olympus, Tokyo, Japan) linked to a semi-automatic particle analyser (ASM 68K, Ernst-Leitz-Wetzlar, Wetzlar, Germany), thanks to the courtesy of Besins Iscovesco Laboratories. There was no change in the particle diameter after the polymer coating and the thickness of the polymer layer was too low to be evaluated by this method.

The specific surface areas were measured by adsorption of nitrogen at − 196°C using the BET method. The ion-exchange capacity of the support was measured by frontal chromatography using 15 m*M* Tris–HNO_3 buffer (pH 7) as the mobile phase and 15 m*M* Tris–HCl as the displacer eluent. The specific surface areas of the supports decrease with increasing amount of polymer coating.

Chromatographic evaluation

The chromatographic experiments were performed on an HPLC system consisting of a pump (HPLC pump 420; Kontron Instruments, Zurich, Switzerland), a sample injector (Model 7125; Shimadzu, Kyoto, Japan) operating at 280 nm).

The silica or diol silica supports coated with QPVI were slurry packed into 7 × 0.41 cm I.D. columns. The temperature of the column was maintained at 20°C during the experiments using a thermostated water-bath. The eluent was 15 m*M* triethanolamine (pH 7). For the frontal analysis the eluents used were 15 m*M* Tris–HNO_3 buffer (pH 7) and 15 m*M* Tris-HCl buffer (pH 7). The amount of protein injected was chosen to be sufficiently small not to overload the column. The plate heights were determined from measurements of peak width at 0.6 of the peak height.

The capacity factor, k', and the linear velocity, u, were calculated by reference to the retention volume of a tracer; u is related to the interstitial velocity, u_e by $u = u_e/(1 + k_0)$, where k_0 is the column permeation ratio of the protein studied. The selected tracer was a polysaccharide (Shodex Standard kit; Sopares, Gentilly, France) of molecular weight (MW) 20 800 g/mol, close to that of the protein studied. The retention volume of the tracer was determined using a differential refractometer (model R401; Waters Assoc., Milford, MA, U.S.A.).

RESULTS AND DISCUSSION

The chromatographic study of the ion-exchange supports was performed by examining both the retention behaviour of α-lactalbumin and the band broadening of its elution peak. This globular protein (MW = 16 000 g/mol, p*I* = 4.8, Stokes radius = 1.95 nm) [20] was chosen as the solute sample, as anion exchangers are often used to separate milk proteins. The quaternized homopolymer QPVI was found to have a high anion-exchange capacity and α-lactalbumin was the first acidic protein to elute in a reasonable retention time for performing systematic chromatographic studies.

Evaluation of retention

The retention behaviour of proteins on ion-exchange supports may be evaluated by using the stoichiometric displacement model. This model, which was first used by Regnier and co-workers [21,22] to describe the variation of the retention volume with salt concentration, was recently extended by Melander *et al.* [23] to include electrostatic and the hydrophobic interactions.

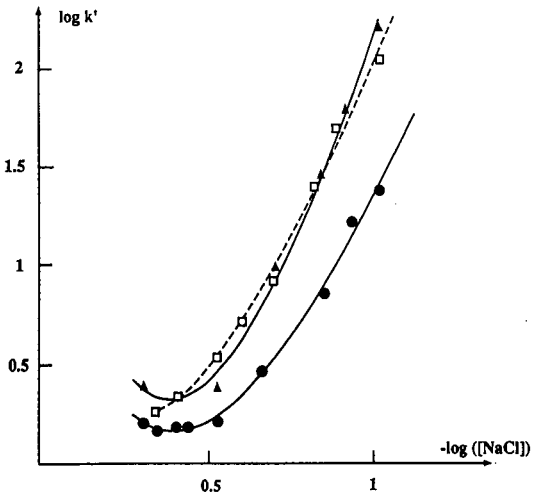

Fig. 1. Dependence of log k' on log (salt concentration). Eluent, 0.015 M triethanolamine buffer with NaCl (pH 7); flow-rate, 1 ml/min; support, silica. Coating method: dashed line, adsorption; solid lines, solvent evaporation. QPVI: □ = 7.6%; ▲ = 8.5%; ● = 3.8%.

A three-parameter equation relates the capacity factor, k', to the concentration of the displacing salt, c_s:

$$\log k' = \alpha - Z \log c_s + \gamma c_s \quad (1)$$

where Z and γ are constants characterizing the electrostatic and hydrophobic interac-

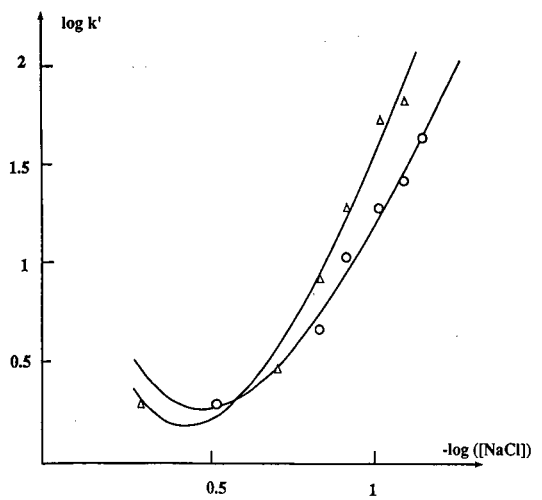

Fig. 2. Dependence of log k' on log (salt concentration). Eluent, 0.015 M triethanolamine buffer with NaCl (pH 7); flow-rate, 1 ml/min; support, diol silica; coating method, solvent evaporation. QPVI: △ = 9%; ○ = 3%.

TABLE II

PARAMETERS OF RETENTION OF α-LACTALBUMIN ON QPVI COATED ON SILICA SUPPORTS

Support	QPVI (%)	α	Z	γ
Silica	7.6[a]	−3.2	4.9	3.9
	8.5[b]	−5.0	6.6	6.8
	3.8[b]	−3.5	4.5	4.8
Diol silica	9.0[b]	−4.7	5.7	6.7
	3.0[b]	−3.7	4.4	5.7

[a] Adsorbed from solution
[b] Coated by solvent evaporation.

tions, respectively; $Z = Z_p/Z_s$, where Z_p is the number of protein charges and Z_s is the valency of the displacing salt.

The variations of k' with the displacing salt concentration are shown in Figs. 1 and 2 for the reticulated PVI stationary phase adsorbed or coated by evaporation on silica or diol silica. Eqn. 1 fits the experimental data well for the plot of log k' *vs.* log c_s, where the hydrophobic effect generates a slight curvature of the lines predicted to be straight from a pure ion-exchange model. The corresponding parameters α, Z and γ are listed in Table II.

Evaluation of band width

The band broadening of the elution peak is characterized from the variation of the plate height, H, with the flow-rate. The plate height is the sum of various increments [24,25]: axial dispersion, eddy diffusion, diffusion into the pores and kinetic mass transfer between the mobile phase and the stationary phase. The last two contributions are proportional to the interstitial velocity, u_e. The plate height increment due to the diffusion into the pores is given by [25]

$$H_i = \frac{d_p^2}{30D_m} \cdot \frac{(k_o + k' + k'k_o)^2}{k_o(1 + k_o)(1 + k')^2} \cdot u \qquad (2)$$

where d_p is the particle diameter and D_m is the diffusion coefficient of the solute inside the porous particle.

The plate-height contribution to solute mass-transfer kinetics in the ion-exchange stationary film [24] is given by the sum of two contributions, corresponding to the diffusion into the stationary phase film H_s:

$$H_s = \frac{k'}{(1 + k')^2} \cdot \frac{qd_f^2}{D_s(1 - \phi)} \cdot u \qquad (3)$$

and the adsorption–desorption process H_k:

$$K_k = \frac{2k'}{(1 + k')^2} \cdot \frac{\phi}{k_d} \cdot u \qquad (4)$$

where D_s is the diffusion coefficient of the protein into the polymer, k_d the desorption rate constant of the ion-exchange process and ϕ is the fraction of retained solute which is adsorbed; the mean depth of the stationary phase units is d_f and the value of the configuration factor q depends on the precise shape of these units. The value of q is 2/3 for a uniform stationary film of thickness d_f and 2/15 for spherical ion-exchange beads, with $d_f = d_p/2$.

At high flow-rates the plate height varies linearly with the velocity u:

$$H = A + Cu \tag{5}$$

In this expression the plate height due to longitudinal diffusion is neglected and the A term roughly approximates the plate height due to eddy diffusion. The term Cu may be considered as approximately equal to the sum $H_i + H_s + H_k$. Evaluating the relative importance of each contribution to the band broadening is difficult. The increment for diffusion into pores may be determined from variations of the particle size [26]. This approach was not considered here because, as shown later from k' variations, the most important cause of efficiency loss was slow mass-transfer kinetics.

The relative importance of the contributions of H_s and H_k to the plate height may be demonstrated from variations of H with the capacity factor. For $k' \gg 1$ the plate height increment H_i due to the diffusion into the pores is almost independent of

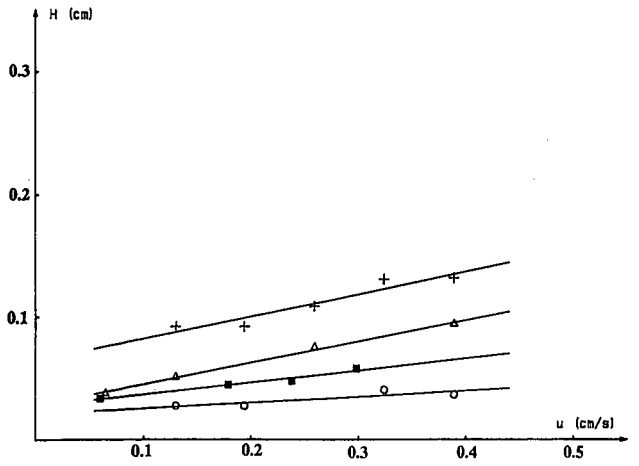

Fig. 3. Influence of capacity factor on plate height for α-lactalbumin eluted from QPVI. H *vs.* u plot [$u = u_c/(1 + k_0)$]. Eluent, 0.015 *M* triethanolamine buffer with NaCl (pH 7); support, 7.6% QPVI adsorbed on silica.

	k'	[NaCl] (m*M*)
+	8	210
△	27	150
■	52	125
○	115	100

k' variations, whereas the sum of H_s and H_k is roughly proportional to $1/k'$. This approach is valid if the other constants producing band broadenings are kept constant while the experimental conditions are modified to obtain a retention variation.

In agreement with eqn. 5, the plate height is a linear function of the mobile phase velocity, as shown in Fig. 3 for the column packed with QPVI adsorbed on silica. The large decrease in the column efficiency, together with the larger slopes C observed for lower k' values, show that the solute mass transfer between the mobile and the stationary phase contributes significantly to the plate-height term.

The efficiencies are low, as the reduced plate heights (H/d_p) lie between 20 and 140. The restricted diffusion of α-lactalbumin into the 100-Å pores of the support used can partially explain [14] the poor efficiencies observed for the protein peak. Moreover, this range is commonly found when dealing with the isocratic elution of proteins in ion-exchange chromatography, as shown by Hearn *et al.* [15], who measured the plate heights for several proteins eluted from a Mono Q anion-exchange resin column.

The influence of k' variations on the plate height was used to determine the most important contributions responsible for the large band broadening observed with the α-lactalbumin peak. Testing the plate-height model with the influence of k' is valid if the overall retention mechanism is kept constant. For given k' values, Hearn *et al.* [15] showed that the nature of the displacer salt influences the mechanism of the protein band broadening. In high-performance affinity chromatography, the influence of k' on plate height was obtained by changing the concentration of the competiting inhibitor in the eluent [13].

In this work, the influence of retention on band broadening was obtained by varying the salt concentration in the mobile phase. The mean time τ spent by the solute in the stationary phase will be considered. Its theoretical expression, τ_{th}, can be derived from the previous plate-height expressions:

$$\tau_{th} = \frac{qd_f^2}{2D_s(1 - \phi)} + \frac{\phi}{k_d} \tag{6}$$

It may be determined experimentally from the slope of the H *vs.* u curves if one can correct it for the mass transfer from the mobile phase to the stagnant fluid. For affinity experiments [26,27], the correction for the diffusional contribution in the stagnant fluid is difficult to determine because of the inacurate calculations of the predicted plate height as a function of k'.

If we assume that the main contribution to the C term is due to the diffusion into the polymer stationary phase film (H_s) and to the adsorption–desorption process (H_k), the experimental value of the mean desorption time, τ_{ex}, can be calculated from

$$\tau_{ex} = (1 + k')^2 \, C/2 \, k' \tag{7}$$

The values of τ_{ex} calculated from the slope C of the straight lines in Fig. 2 are listed in Table III. Within experimental error, the τ_{ex} measurements (2.7 s) are independent of k' variations in the range $27 < k' < 115$. This confirms the assumption that for large k' values one can neglect in the C term the contribution of the restricted diffusion into the pores. Moreover, these results show that the mechanism responsible

TABLE III

RESULTS OF MASS-TRANSFER KINETIC MEASUREMENTS

Support	QPVI (%)	[NaCl] (*M*)	k'	C (s)	τ_{ex} (s)
Silica	7.6[a]	100	115	0.05	2.7
		125	52	0.11	3.0
		150	27	0.17	2.5
		210	8	0.20	1.1
	8.5[b]	125	63	0.48	16.0
	3.8[b]	75	62	0.09	2.9
Diol	9.0[b]	100	57	1.6	47.0
silica	3.0[b]	85	42	0.38	8.3

[a] Adsorbed from solution.
[b] Coated by solvent evaporation.

for the band broadening of α-lactalbumin is not modified by varying the salt concentration in the range 0.1–0.15 *M* NaCl. Slow mass transfers between the liquid phase and the stationary phase are therefore mainly responsible of the loss of efficiency observed at higher flow-rates. These effects include diffusion in the polymer film (H_s term) and the adsorption–desorption process on the ion-exchange site (H_k term).

For smaller capacity factors ($k' = 8$), a deviation from the plate-height model predictions was observed since $\tau_{ex} = 1$ s. This effect may be due to the additional retention mechanism observed at higher salt concentrations and caused by an increase in the hydrophobic effect.

Karger and co-workers [28–30] have shown from fluorescence and chromatographic measurements that conformational changes of α-lactalbumin can occur on hydrophobic surfaces from the folded to the unfolded protein state. These effects, which increase with increasing degree of hydrophobicity of the stationary phase and temperature, may lead to additional broadenings when the elution peaks of the conformers overlap. One cannot exclude the occurrence of such a mechanism in the present experiments carried out at 20°C, although the ion-exchange surface is weakly hydrophobic.

To evaluate the effect of film thickness, given amounts of polymer were deposited on the silica support by evaporation of the solvent. A poor coating was obtained by this method, as shown in Fig. 4, where the efficiencies of α-lactalbumin on the supports prepared by polymer adsorption from the solvent or by evaporation of the solvent are compared. For about the same amount of polymer coated on silica and the same k' value, a large decrease in the column efficiency is observed when the polymer is coated by evaporation. No variation in the particle size was observed with silica supports coated with large amounts of polymer and the poor characteristics of the support coated by evaporation are close to those of the support coated by adsorption, as shown from surface area measurements (Table I).

Hence the poor efficiency observed with α-lactalbumin eluted from the support coated by evaporation is due to the sum of the H_s and H_k contributions, as the slope of the straight line (H *vs.* u) is five times larger for the same amount of PVI on silica

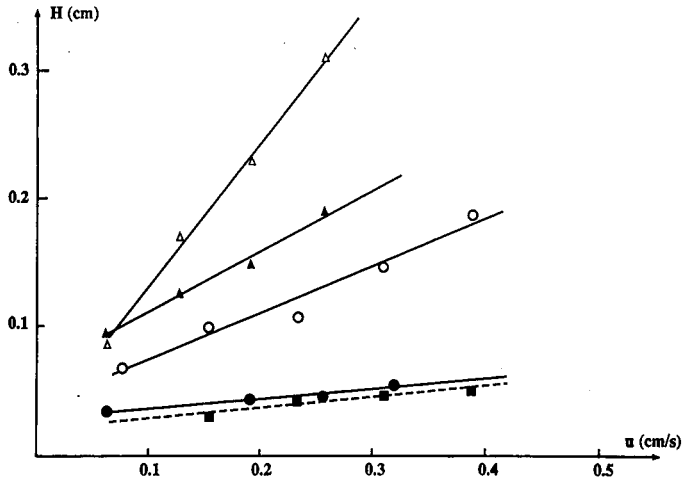

Fig. 4. Comparison of the band broadening of α-lactalbumin eluted from various QPVI coatings. *H vs. u* plot [$u = u_e/(1 + k_0)$]. Eluent, 0.015 *M* triethanolamine buffer with NaCl (pH 7). Coating method: dashed line, adsorption; solid lines, solvent evaporation.

	QPVI (%)	Support	k'	[NaCl] (m*M*)
■	7.6	Silica	63	125
▲	8.5	Silica	52	125
●	3.8	Silica	62	100
△	9.0	Diol silica	56	100
○	3.0	Diol silica	42	85

(Fig. 4). This loss of efficiency observed with the polymer deposited by evaporation is probably due to the formation of several layers taking place in units which are not uniformly distributed on silica.

With a support coated with a smaller amount of polymer by evaporation (3.8% PVI), an improved efficiency was observed (Fig. 4), with a value of τ_{ex} now close to that obtained with the adsorbed polymer (Table III). The 3.8% and 8.5% PVI supports were both obtained by solvent evaporation. The coatings are similar and the ratio of the mean stationary film thickness is probably close to 2.2, leading to an H_s contribution that is 4.8 times greater with the large PVI coating. Therefore, the solute diffusion into the polymer film mainly contributes to the large increase in the plate heights observed at high flow-rates and large percentages of PVI coated by evaporation.

The retention volume was measured as a function of the displacing salt concentration for various amounts of polymer coatings applied on the silica support (Fig. 1). Larger polymer coatings produce higher ion-exchange capacities (Table I) and therefore higher α-lactalbumin capacity factors. For similar experimental conditions, the retention volume on QPVI adsorbed on silica is close to that observed with approximately the same amount of polymer deposited by evaporation. The parameters of eqn. 1 calculated by fitting the model to the plot of log k' *vs.* log [NaCl] are listed in Table II. The parameters Z and γ are larger on the polymeric phase having a poor

coating layer, obtained by solvent evaporation. An increase in the number of protein charges is then observed ($Z_s = 1$ and $Z = Z_p$) with an increase in the degree of hydrophobicity of the stationary phase.

With the adsorbed polymeric coating the hydrophobic properties ($\gamma = 3.9$) are even lower than those observed with a stationary phase having half the amount of polymer but coated by evaporation ($\gamma = 4.8$). These results show that stationary phases with faster mass-transfer exchanges and lower hydrophobic properties are obtained when the polymer film has a smaller thickness and is more uniformly distributed on the support.

The role of the solid support was studied by comparing the chromatographic properties of silica coated with QPVI with those of a diol silica coated with the same polymer. The diol support is fairly inert and does not have high adsorption properties. Therefore, coating the polymer by the adsorption procedure was not possible and the polymer coating was achieved by evaporation of the solvent.

The same trends as were found with porous silica were observed when various amounts of QPVI are deposited on diol silica: slower mass-transfer exchanges and greater hydrophobic character for larger amounts of polymer coated from solvent evaporation (Tables II and III). In Fig. 4 the steeper lines at higher percentage of QPVI on diol silica reveal a slow diffusion effect in the polymer stationary phase.

The efficiency of the column packed with the diol silica support coated with QPVI is lower than that observed with the corresponding silica column having about the same amount of polymer deposited by solvent evaporation (Fig. 4). The results in Table III show that the mass transfer term τ_{ex} is roughly three times larger with a polymer layer deposited on diol silica. The coating of QPVI on diol silica is therefore less uniform with stationary film units of mean depth roughly 1.7 times larger than those on silica. These results show the importance of the role of the solid support for achieving thin films of uniform thickness: the contribution of the slow diffusion process in the stationary phase increases with increasing non-uniformity of the coating and thickness of the polymer layer.

However, one cannot exclude a contribution of the slow adsorption–desorption kinetics, which were found in affinity systems to be mainly responsible for the poor efficiencies for the isocratic elution of proteins [13,14]. It is in fact difficult to differentiate between the H_s and H_k terms as the Z values increases with increasing amount of polymer deposited by evaporation. This result indicates a larger number of contact points of the protein with the support, which may lead to slower desorption kinetics with a larger contribution of the H_k term. Compared with the silica support coated by evaporation, the slope C of the H *vs.* u curve is 3–4 times larger with diol silica (Table III), but similar Z values are observed for comparable amounts of polymer deposited on silica by evaporation (Table II). This shows that the diffusion mechanism dominates with the diol silica support and probably with the silica support coated by evaporation with a large amount of polymer.

CONCLUSIONS

At high flow-rates the most important contribution to the band broadening of the α-lactalbumin elution peak is the slow mass transfer between the mobile phase and the stationary phase. On the basis of a sole band-broadening analysis, it is not

possible to distinguish between the contributions of the diffusion of the protein into the stationary phase and the conformational changes. A fluorescence study of the kinetic mechanism [26] would be helpful for determining the relative importance of this last effect.

However, in ion-exchange chromatography, slow diffusion which may lead to protein conformational changes is a rate-controlling step and partially explains the low efficiencies observed in isocratic elution with non-uniform coatings of large thickness: the H_s increment given by eqn. 3 increases strongly with increase in ϕ, the ion-exchange adsorption fraction which is always close to 1 in ion-exchange chromatography. It is therefore important, as shown experimentally in this work, to achieve uniform polymer coatings of small thickness in order to increase the efficiencies and to reduce the degree of hydrophobicity of the stationary phase.

The role of the supporting material was demonstrated, as the best efficiencies were obtained by adsorbing the polymer on porous silica. The high concentration of SiOH permits an irreversible adsorption of the functional groups of the polymer. This produces a more uniform coating and a thinner film. It is therefore to be expected that selecting an inert or a deactivated matrix will produce a poor polymer coating with a high degree of hydrophobicity that may lead to protein conformational changes.

This paper was limited to the study of the chromatographic properties of α-lactalbumin. However, the same adsorption technology on silica supports is now used with weaker anion exchangers based on PVI copolymers [31] and gives satisfactory elution behaviours with good protein recoveries.

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Determination of several retinoids, carotenoids and E vitamers by high-performance liquid chromatography

Application to plasma and tissues of rats fed a diet rich in either β-carotene or canthaxanthin

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ABSTRACT

A method, using two different systems, is described for the high-performance liquid chromatographic analysis of retinol, retinal, retinoic acid, retinyl acetate, retinyl palmitate, α-, β- and γ-carotene, β-apo-6′-, β-apo-8′-, β-apo-10′- and β-apo-12′-carotenal, ethyl β-apo-8′-carotenoate, α-tocopherol and α-tocopheryl acetate. The first system consists of a laboratory-packed Hypersil-ODS 3-μm column and a mobile phase of acetonitrile–methylene chloride–methanol–water (70:10:15:5, v/v). The second system consists of a laboratory-packed Nucleosil C_{18} 3-μm column and a mobile phase of acetonitrile–0.1 *M* ammonium acetate (80:20, v/v). The detection limits in standard solutions were 10 ng/ml for retinoids and carotenoids and 60 ng/ml for the E vitamers. Analysis of the tissues and plasma of rats, after 2 weeks on a diet supplemented with either β-carotene or canthaxanthin (both 2 mg/g), led to the conclusion that the rats were able both to transport and store β-carotene and canthaxanthin and to convert β-carotene to retinol. Incubation of cytosol preparations from the mucosa of the small intestine of rat with 1 μg of β-carotene resulted in the formation of 10–20 ng of retinal within 1 h.

INTRODUCTION

Vitamin A and β-carotene may be important agents in the prevention of cancer [1–3]. After absorption by the small intestine, most β-carotene is believed to be converted to vitamin A, while a small part may be transported as β-carotene [4]. It is not clear whether β-carotene is protective after conversion to vitamin A, or by itself, for instance as an antioxidant [5].

The primary interest is in the absorption of β-carotene in the intestine and its

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possible cleavage following absorption. Two cleavage theories exist: the first assumes a central cleavage by 15,15′-dioxygenase, resulting in two molecules of retinal, whereas the second assumes a random cleavage, resulting in retinal, various β-apo-carotenals and other products.

To validate these two theories it should suffice to measure the amount of retinal formed from β-carotene. However, the experience is that, under experimental conditions *in vitro*, the amount of retinal formed is too small to confirm the first theory [6]. To confirm the second theory products other than retinal (and not originating from retinal) should be demonstrated.

It is therefore important to measure the possible products resulting from random β-carotene cleavage. Several high-performance liquid chromatographic (HPLC) procedures have already been described for the determination of retinol, retinoic acid, retinyl esters and/or carotenoids [7–9]. Furr [10] described a method for the analysis of retinal, among other retinoids, using a gradient programme. Hansen and Maret [11] measured retinoids, carotenoids and β-apo-carotenals using three different HPLC systems.

In this paper a method is described for the HPLC analysis (using two different systems) of retinol, retinal, retinoic acid, retinyl acetate, retinyl palmitate, α-, β- and γ-carotene, β-apo-6′-, β-apo-8′-, β-apo-10′- and β-apo-12′-carotenal, ethyl β-apo-8′-carotenoate, α-tocopherol and α-tocopheryl acetate. In addition, two applications of the method are described. The first is the analysis of tissues obtained from female rats fed a diet enriched with either β-carotene or canthaxanthin. The second is the analysis of samples of an *in vitro* cleavage assay with intestinal cytosol preparations, described as the 15,15′-dioxygenase assay by Goodman *et al.* [12].

EXPERIMENTAL

Chemicals

β-Apo-6′-caretenal was a gift from BASF (Arnhem, The Netherlands). Retinyl stearate, γ-carotene, β-apo-10′-carotenal and β-apo-12′-carotenal were gifts from Hoffmann-La Roche (Mijdrecht, The Netherlands). β-Carotene was obtained from Merck (Darmstadt, Germany). β-Apo-8′-carotenal, ethyl β-apo-8′-carotenoate and canthaxanthin were obtained from Fluka (Buchs, Switzerland). Other reference compounds were purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile (HPLC grade) from Westburg (Leusden, The Netherlands) was used. All other chemicals were of analytical-reagent grade.

Chromatography

HPLC analyses were performed using a system incorporating a Gynkotek 300 C constant-flow pump (Kipp Analytica, Delft, The Netherlands), an ISS-100 automatic injector with cool tray (Perkin-Elmer, Gouda, The Netherlands) and two programmable 783 absorbance detectors (Applied Biosystems, Rotterdam, The Netherlands). Two stainless-steel Hyperchrome HPLC columns (125 mm × 4.6 mm I.D.) were packed in the laboratory with Hypersil ODS 3 μm (Shandon Southern Products, Astmoor, U.K.) or Nucleosil 120-3 C_{18} (Machery-Nagel, Düren, Germany) by the balanced-density slurry technique on a column-packing installation designed at the TNO Toxicology and Nutrition Institute, using a Haskel DSTV-150 pump

(Ammann Technik, Stuttgart, Germany). The slurry and packing solvents were isopropanol and methanol, respectively. Elution profiles were displayed on a Kipp BD 41 recorder (Kipp Analytica).

The mobile phase used for the Hypersil column consisted of acetonitrile–methylene chloride–methanol–water (70:10:15:5, v/v). A flow programme of 0.5–2.0 ml/min was used (0–13 min at 0.5 ml/min; 14–24 min at 1.0 ml/min; 25–46 min at 1.5 ml/min; 47–57 min at 2.0 ml/min; 58–60 min at 0.5 ml/min). The Nucleosil column was eluted at a flow-rate of 1.0 ml/min with acetonitrile–0.1 *M* ammonium acetate (80:20, v/v).

Detection after separation on the Hypersil column was carried out using one detector set at 350 nm and a second detector switching after 16 min from 445 to 292 nm and returning 9 min later to 445 nm for the last part of the HPLC run.

Procedures

Standard solutions were prepared in methanol. The preparation of standard solutions and the extraction of plasma and tissue samples and of incubation mixtures from the dioxygenase assay were carried out under subdued light. The actual concentrations of the standards were determined by measuring the absorbance of diluted stock solutions using a Ultrospec K spectrophotometer (LKB, Cambridge, U.K.) and calculating the concentrations based on published spectral data [13–15].

Livers were dismembrated in liquid nitrogen using a Mikro-dismembrator II (Braun, Melsungen, Germany). All other tissues were homogenised with an Ultra-Turrax homogeniser (Wilten Woltil, De Bilt, The Netherlands). Samples were homogenised in brown-coloured test-tubes with 3 ml of doubly distilled water containing EDTA (10 m*M*), ascorbic acid (1 mg/ml) and acetic acid (1%, v/v). Plasma samples of 100 μl were mixed with 100 μl of 0.9% (w/v) NaCl. To precipitate proteins, 2 ml (0.2 ml for plasma samples) of methanol [containing 1 mg of butylated hydroxytoluene (BHT) per ml added as an antioxidant] were added and the mixture was vortexed for 30 s. After 10 min, 4 ml (0.4 ml for plasma) of chloroform (containing 1 mg/ml BHT) were added and the sealed tubes were vortexed for 4 min. After centrifugation the chloroform layer was separated and evaporated under nitrogen. The residue was dissolved in methanol, transferred into brown HPLC injection vials and placed in the HPLC tray of the injector, which was cooled down to about +4°C to increase stability, especially of the aldehyde forms of the compounds of interest.

For the incubation mixtures of the dioxygenase assay the same procedure was followed, except that ethanol was used to precipitate the proteins and hexane was used for extraction. The residues were dissolved in water-free eluent.

RESULTS AND DISCUSSION

Characteristics of the high-performance liquid chromatographic methods

With the Hypersil column the HPLC method described here yielded a complete resolution of retinol, retinyl acetate, retinyl palmitate, α-, β- and γ-carotene, β-apo-6′-, β-apo-8′-, β-apo-10′- and β-apo-12′-carotenal, ethyl β-apo-8′-carotenoate, α-tocopherol and α-tocopheryl acetate. The method using the Nucleosil column gave a complete resolution of retinoic acid, retinal, retinol and retinyl acetate. It was not possible to separate all the compounds in a single run using the Hypersil column;

retinoic acid eluted in the void volume and no baseline separation could be obtained for retinol, retinal and retinyl acetate. Separation could be improved by increasing the polarity of the eluent. However, this extends the elution time of β-carotene by several hours. As the detector was used in a very sensitive setting, gradient elution was not useful because of a rising baseline. Flow programming shortened the run to 45 min.

Using the Hypersil column two spectrophotometric detectors were needed as the time between the elution of the retinyl acetate and β-apo-12′-carotenal and the time between the elution of the β-carotene and retinyl palmitate were too short for reliable wavelength switching. This is why the carotenoids and tocopherols were measured

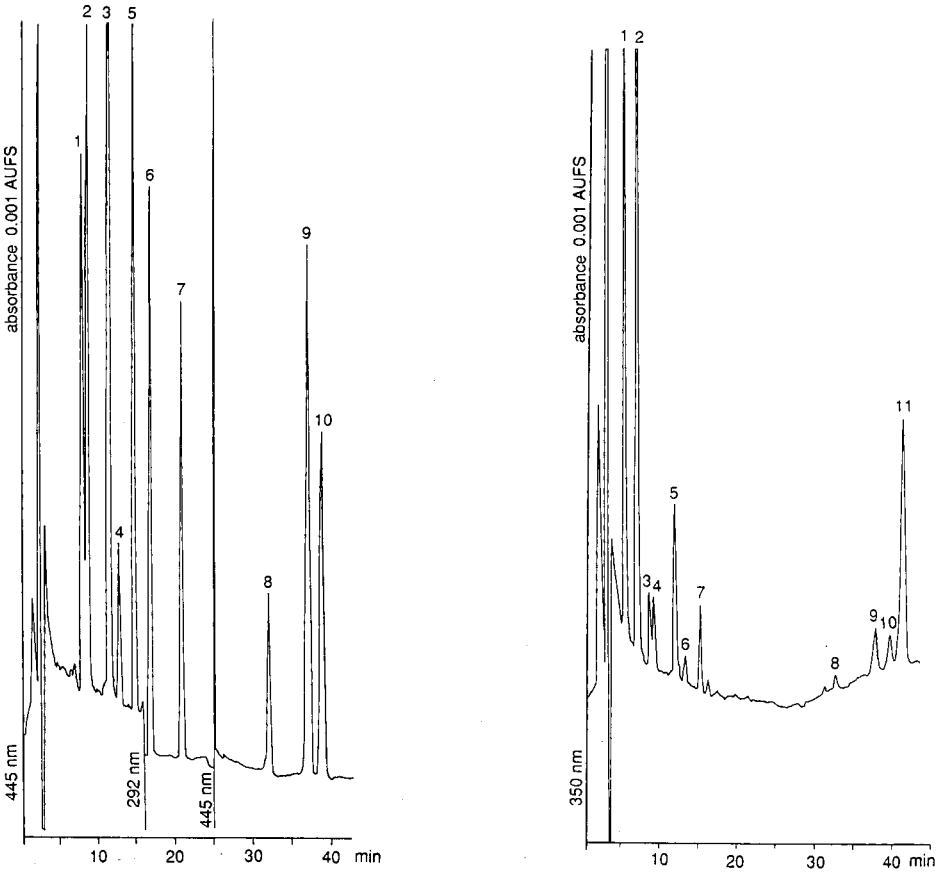


Fig 1. HPLC elution profile of a mixture of reference compounds after injection onto the Hypersil column and detection at a wavelength switching from 445 to 292 nm and back to 445 nm. Peaks: 1 = β-apo-12′-carotenal; 2 = β-apo-10′-carotenal; 3 = β-apo-8′-carotenal; 4 = β-apo-6′-carotenal; 5 = ethyl β-apo-8′-carotenoate; 6 = α-tocopherol; 7 = α-tocopheryl acetate; 8 = γ-carotene; 9 = α-carotene; 10 = β-carotene.

Fig. 2. HPLC elution profile of a mixture of reference compounds after injection onto the Hypersil column and detection at a wavelength of 350 nm. Peaks: 1 = retinol; 2 = retinyl acetate; 3 = β-apo-12′-carotenal; 4 = β-apo-10′-carotenal; 5 = β-apo-8′-carotenal; 6 = β-apo-6′-carotenal; 7 = ethyl β-apo-8′-carotenoate; 8 = γ-carotene; 9 = α-carotene; 10 = β-carotene; 11 = retinyl palmitate.

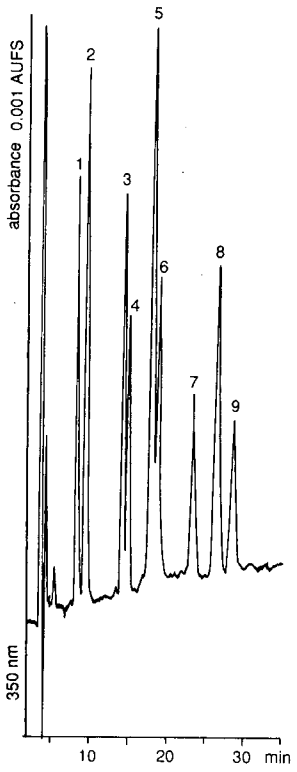


Fig. 3. HPLC elution profile of a mixture of reference compounds after injection onto the Nucleosil column and detection at 350 nm. Peaks: 1 = 13-*cis*-retinoic acid, 2 = all-*trans*-retinoic acid; 3 = 13-*cis*-retinol; 4 = all-*trans*-retinol; 5 = 9- and 13-*cis*-retinal; 6 = all-*trans*-retinal; 7 = etretinate; 8 = 13-*cis*-retinyl acetate; 9 = all-*trans*-retinyl acetate.

with one detector switching from 445 to 292 nm and back to 445 nm (see Fig. 1 for an elution profile), whereas retinol, retinyl acetate and retinyl palmitate were determined using a second detector set at 350 nm (Fig. 2). Fig. 3 shows the results of the separation of a standard retinoid mixture on the Nucleosil column.

The use of BHT as an antioxidant in the extraction procedure resulted in a BHT peak on the Nucleosil column with a retention time of 6 min, which did not disturb the measurements. However, on the Hypersil column BHT eluted after 11 min, and hence interfered with the peaks of β-apo-10′-carotenal and β-apo-8′-carotenal. In a first trial without BHT no negative effects were found, therefore in further studies BHT was not used if the presence of β-apo-carotenals was expected in a sample.

To calculate recoveries, liver samples to which known amounts (comparable to endogenous levels) of β-carotene, retinoic acid, retinol and retinal had been added were analysed in duplicate together with untreated samples. Recoveries, (mean $\pm$ S.D.) were 102.5 $\pm$ 0.7% for β-carotene, 102.5 $\pm$ 10.6% for retinoic acid, 95.5 $\pm$ 4.9% for retinol and 95.5 $\pm$ 6.4% for retinal.

Assuming that the signal-to-noise ratio should be at least 3, and using an injection volume of 50 μl, the detection limits of the method correspond to a 10 ng/ml

standard solution for retinoids and carotenoids and 60 ng/ml for α-tocopherol and α-tocopheryl acetate.

Using the method described it is possible to measure more compounds than, to the authors' knowledge, are described in the literature, using not more than two systems.

Application to tissue samples

Two groups, each of five female Wistar rats (post-weaning) were fed regular laboratory feed (containing 2 mg/g retinol) supplemented with either 2 mg/g β-carotene or 2 mg/g canthaxanthin (no pro-vitamin A activity). β-Carotene and canthaxanthin were added as beadlets (a gift from Hoffmann-La Roche, Basle, Switzerland). The rats had free access to food and water and consumed around 10 g of food per day.

After 2 weeks the rats were sacrificed. The animals were anaesthesized with diethyl ether, blood was collected by cardiac puncture and whole body perfusion with 0.9% (w/v) NaCl was carried out before the tissues were collected. The tissues were immediately frozen in liquid nitrogen and stored at −80°C. Plasma was separated from the blood and also stored at −80°C.

Retinyl stearate and canthaxanthin were measured using the Hypersil column. Retinyl stearate was detected at 350 nm and eluted about 10 min after retinyl palmitate. Canthaxanthin was detected at 445 nm and eluted at the same time as β-apo-10′-carotenal. As canthaxanthin and β-apo-10′-carotenal were not expected to be present together in the samples, this was no real problem. In fact, by using more water in the eluent, canthaxanthin can be separated from the apocarotenals.

Figs. 4 and 5 show the chromatographic traces of a lung sample analysed on the Hypersil column. In this example the detection was carried out with one detector set at 445 nm and the second switching from 350 to 292 nm, then returning to 350 nm. The Nucleosil column was used to determine retinoic acid.

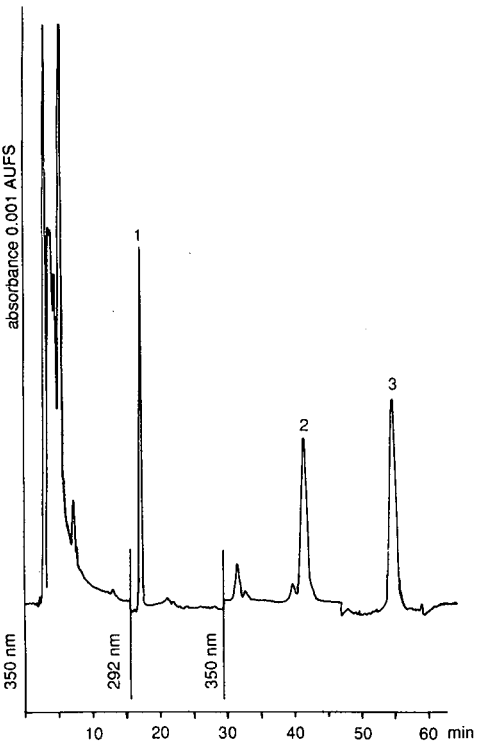
Table I shows the results of the plasma and tissue analyses. Variable amounts of β-carotene and canthaxanthin were stored in subcutaneous fat in which 1–105 μg/g β-carotene and 2–113 μg/g canthaxanthin were found.

Apocarotenals could not be confirmed in any sample. In liver and lung samples some indications for apocarotenals were found, but these did not exceed the detection limits.

From Table I it can be concluded that the rats were able to convert β-carotene to retinol, as the rats fed β-carotene showed higher levels of retinol in liver, lung and mamma than rats fed canthaxanthin. Furthermore, β-carotene was found in tissues, so the rats were also able to transport unconverted β-carotene to the liver, lung and mamma. Canthaxanthin was also transported to these organs.

Application to 15,15′-dioxygenase assay samples

The proximal 60 cm of the small intestine was removed from male or female Wistar rats and flushed with ice-cold 0.9% NaCl. The mucosa was scraped off in 0.1 *M* potassium phosphate buffer and cytosol was isolated by differential centrifugation. Incubation was carried out as described by Goodman *et al.* [12] with 200 μl of cytosol (4 mg of protein), 0.125 g/l α-tocopherol and 1 μg of β-carotene in 0.1 *M* potassium phosphate buffer, pH 7.7, containing 15 m*M* nicotinamide, 2 m*M* $MgCl_2$, 5 m*M*



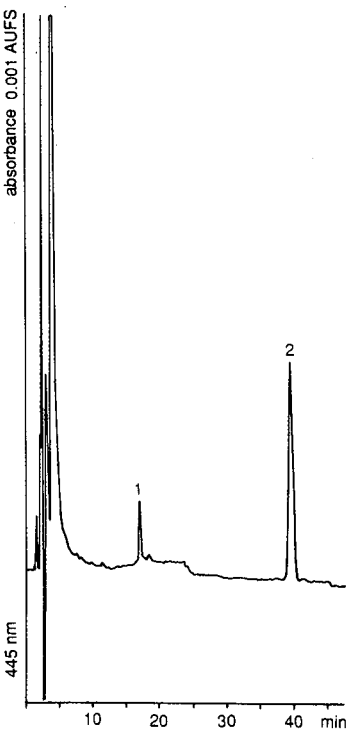


Fig. 4. HPLC elution profile of a lung sample after injection onto the Hypersil column and detection at a wavelength switching from 350 to 292 nm and back to 350 nm. Peaks: 1 = α-tocopherol; 2 = retinyl palmitate; 3 = retinyl stearate.

Fig. 5. HPLC elution profile of a lung sample after injection onto the Hypersil column and detection at a wavelength of 445 nm. Peaks: 1 = α-tocopherol; 2 = β-carotene.

TABLE I

RETINOID AND CAROTENOID CONCENTRATIONS IN PLASMA AND TISSUES OF RATS

Rats had been fed a diet containing either 0.2% β-carotene (group 1) or 0.2% canthaxanthin (group 2) for 2 weeks.

Compound	Plasma (μmol/l)		Liver (μg/g)		Lung (μg/g)		Mamma (μg/g)	
	1	2	1	2	1	2	1	2
Retinol	0.62	0.63	7.8	1.3	3.61	0.67	0.38	0.09
Retinyl palmitate	–	–	395	48	18.9	5.6	0.63	–
Retinyl stearate	–	–	61	8.5	8.15	2.75	–	–
Retinoic acid	–	–	0.09	–	0.03	–	0.05	0.01
β-Carotene	0.33	–	51	0.35	2.10	–	0.09	
α-Carotene	–	–	1.19	0.08	–	–	–	–
Canthaxanthin	–	0.73	–	294	–	6.44	–	2.38
α-Tocopherol	24.4	37.8	61.6	53.0	28.7	37.3	36.7	22.6

glutathione, 1.7 m*M* sodium dodecyl sulphate and 0.2 g/l L-α-phosphatidylcholine. After 1 h incubation with 1 μg of β-carotene, 10–20 ng of retinal were formed. To optimise the measurement of retinal, the wavelength can be switched to 380 nm. No other products could be demonstrated, although only about 80% of the added β-carotene was left. More experiments are in progress to optimise the assay and to find out whether other products are formed and whether the β-carotene is partially lost during the procedure.

From the results obtained, no definite conclusion can be drawn as to the correct β-carotene cleavage theory. To study the β-carotene cleavage further, experiments with intestinal cell lines are currently being undertaken and *in vivo* experiments with rats are being planned.

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CHROMSYMP. 2127

Application of high-performance liquid chromatography to the analysis of niacin and biotin in Italian almond cultivars

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ABSTRACT

An extraction method involving heat sonication with simultaneous high-performance liquid chromatographic separation of water-soluble vitamins from almonds is described. For the removal of interfering compounds a clean-up procedure on a strong cation-exchange column is suggested. Recovery data after the complete procedure for the six water-soluble vitamins, as well as qualitative and quantitative data for various Italian cultivars of almonds are reported. The method is suitable for the routine evaluation of niacin and biotin.

INTRODUCTION

Almonds are an important crop in Southern Italy, but the numerous cultivars grown vary widely in yield and composition. This is a drawback for the development of Italian almond production. With the aim of screening cultivars for agronomic, technological and nutritional features, to find those most suitable for growth, a research project has been granted funding by the Development Fund for the South of Italy (CASMEZ).

Among the nutritional characteristics to be evaluated, water-soluble vitamin content is of interest, as almonds are considered a good source of B-complex vitamins, mainly niacin (1–10 mg per 100 g), biotin (10–100 μg per 100 g) and thiamin (10–100 μg per 100 g) [1].

Data in the literature are scarce and usually do not specify the cultivar or its origin, or the analytical methods [2]. Therefore we propose that systematic research on water-soluble vitamin content of Italian cultivars should be undertaken. As for the method of choice, the capability for simultaneous multi-vitamin analysis offered by high-performance liquid chromatography (HPLC) caught our attention. This capability, together with its precision, accuracy, specificity and speed, is generally recognized (as also pointed out by Eitenmiller [3]), and is one of the main reasons for the tremendous developments in HPLC methods applied to the analysis of these compounds [4–9]. However, the possibility of using HPLC for the analysis of B-complex vitamins in oil seeds such almonds has not been sufficiently investigated, as the above-quoted reviews show. The preliminary defatting step for almonds was used, as it was

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previously adopted for highly fatty products such as chocolate, almonds and peanuts by Hurst *et al.* [10] for the analysis of thiamin.

EXPERIMENTAL

Apparatus

A Jasco BIP-I liquid chromatograph was used, equipped with a Rheodyne 7125 variable-loop injection valve fitted on a stainless-steel column (33 × 4.6 mm I.D.) prepacked with 3-μm Supelcosil LC-8-DB (Supelco Cat. No. 5-8976) plus a guard column (20 × 4.6 mm I.D.) packed with the same stationary phase, placed in a Dani HPLC oven and connected to a Jasco 850 UV variable-wavelength detector coupled with a Shimadzu Chromatopac C-R1B data processor.

Materials

Standard solutions (0.02, 0.05 and 0.1%) of thiamin, riboflavin, folic acid, pyridoxine, biotin and niacin were prepared dissolving standard compounds (Serva) in 0.1 *M* HCl. The 5 m*M* hexanesulphonic acid sodium salt–0.1% triethylamine solution was brought to pH 2.8 with 75% phosphoric acid and filtered through a Millipore HA 0.45-μm membrane prior to the addition of methanol [11]. The mobile phase was degassed by sonication under vacuum. Before HPLC analysis the standard solutions were filtered through a Millex GV 0.22-μm unit (Millipore).

All the other reagents were of analytical grade.

Methods

Sample preparation. All the samples were previously de-fatted with *n*-hexane by extraction of the ground almonds in a Soxhlet apparatus for 6 h; 30 g of de-fatted almonds, ground to a 0.2-mm powder in a hammer mill, were placed into a 300-ml flask; after having moistened the powder with 10 ml of distilled water, 70 ml of 0.1 *M* HCl were added. The mixture was then sonicated at 75°C for 15 min and, after shifting the pH to 4.6 by adding 0.1 *M* NaOH, transferred into a 100-ml volumetric flask and brought to the mark with water. This extract was filtered through a Gooch filter with Whatman No. 4 paper.

Then, 50 ml of the extract were filtered on a Millipore HA 0.45-μm membrane filter; the vitamins were adsorbed on an aromatic sulphonic acid extraction column (strong cation exchange) (Baker-10-SPE), previously conditioned by passing 6 ml of methanol and 6 ml of water.

After washing (6 ml of water, 6 ml of methanol, 6 ml of water), the retained compounds were eluted from the column with 4.5 ml of 2 *M* KCl–methanol (60:40) solution previously warmed to 70–75°C.

The eluate was collected into a 5-ml volumetric flask and after cooling, brought to volume with the mobile phase.

Prior to injection, the solution was filtered through a Millipore GV 0.22-μm membrane filter.

The whole sample preparation was performed under half-light.

HPLC analysis. Separation was achieved at 35°C according to the following parameters: Stationary phase: Supelcosil LC-8-DB 3 μm; mobile phase: 5 m*M* hexanesulphonic acid sodium salt containing 0.1% triethylamine (pH 2.8)–methanol

(85:15); flow-rate: 1.0 ml/min; detection: 200 nm at 0.08 a.u.f.s. range; sample volume: 10 μl; average retention times: niacin 1.18 min, pyridoxine 1.74 min, thiamin 3.08 min, biotin 4.09 min, riboflavin 5.39 min, folic acid 7.09 min.

RESULTS AND DISCUSSION

For the HPLC separation of the six water-soluble vitamins (thiamin, riboflavin, folic acid, pyridoxine, niacin and biotin) which are reported to be present in almonds [12,13], we applied the conditions suggested by Supelco [11] for the chosen stationary phase (LC-DB-8), specifically made for the fast routine analysis of water soluble vitamins in pharmaceutical products such as multi-vitamin tablets. This column is a deactivated phase which, according to the manufacturer, should provide a best peak shape and a better separation of the vitamins than the standard RP-8 column. The same column was used by Bonomi *et al.* [14] for feed premixes.

Fig. 1 shows a typical separation of a standard mixture containing equal amounts (0.05%) of each vitamin. As almond seeds contain high amounts of lipids (50–65% or more according to the cultivar), prior to the water-soluble vitamin extraction step it was necessary to de-fat the samples by means of a Soxhlet extraction with *n*-hexane. According to Hurst *et al.* [10] the de-fatting treatment should not affect the recovery of B-complex vitamins.

Optimal conditions for acid digestion by means of heat sonication was tested both for the temperature (from 60 to 90°C) and for the time (from 5 to 60 min) required to achieve the highest extraction yield for the least time of digestion. The most suitable conditions were found to be 75°C for 15 min. Increases in temperature and time beyond these points did not noticeably affect the extraction yield.

Since no enzymatic hydrolysis treatments were performed in order to simplify the method, the interfering components overlapping the peaks of the vitamins to be

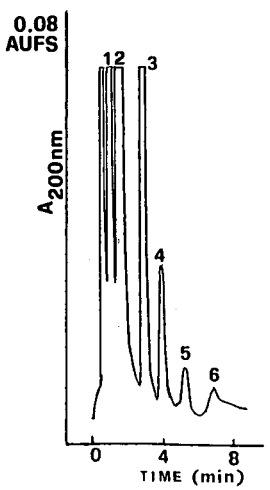

Fig. 1. HPLC separation of 0.05% standard solution containing six water-soluble vitamins. Peaks: 1 = niacin; 2 = pyridoxine; 3 = thiamin; 4 = biotin; 5 = riboflavin; 6 = folic acid. For HPLC conditions see Experimental.

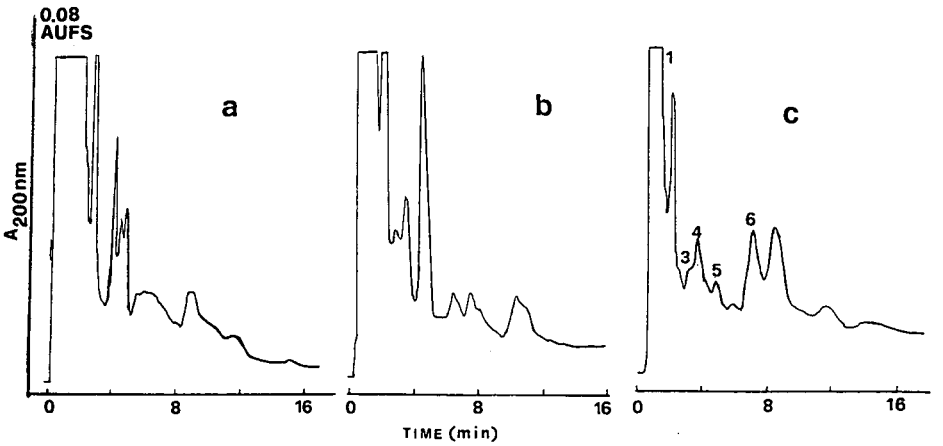

Fig. 2. Effect of clean-up procedure on the HPLC analysis of a *Filippo ceo* c.v. almond extract. (a) Crude extract; (b) removed interfering substances; (c) cleaned-up vitamins. Peaks: 1 = niacin; 3 = thiamin; 4 = biotin; 5 = riboflavin; 6 = folic acid.

quantified (Fig. 2a) were removed by clean-up on a strong cation-exchange phase. We used the method reported by Ayi and co-workers [15,16] for infant formula, with slight modifications as described in the Experimental section. It appears possible to achieve the removal of almost all interference (Fig. 2b) from the water-soluble vitamins (Fig. 2c).

However, only for niacin and biotin did we obtain resolution of the peak which was sufficient to make their quantification possible; for the other vitamins (*i.e.* riboflavin, thiamin, folic acid and pyridoxine) it was only possible to give an estimation of their presence as relative abundance in the extract. In the cleaned-up extract the identification of each vitamin was performed by injecting the same extract spiked with 0.2% standard slution.

The calibration equations for niacin and biotin, over the range 2–10 μg of vitamin, were: $y = 35.06\,x + 2.649$ for niacin, and $y = 24.46\,x + 4.893$ for biotin.

As for the recovery of each vitamin after complete sample preparation (Table I), tests made on 0.1% standard solutions gave good results for niacin, riboflavin and thiamin, while the other three vitamins showed lower recoveries ranging from 79.2%

TABLE I

RECOVERY DATA FROM 0.1% STANDARD SOLUTION SUBMITTED TO THE COMPLETE SAMPLE PREPARATION PROCEDURE (AVERAGE OF FOUR TESTS)

Vitamin	Recovery (%)
Biotin	79.8 ± 8.92
Niacin	97.6 ± 1.01
Riboflavin	92.8 ± 2.05
Thiamin	96.5 ± 1.06
Pyridoxine	85.7 ± 6.42
Folic acid	79.2 ± 8.75

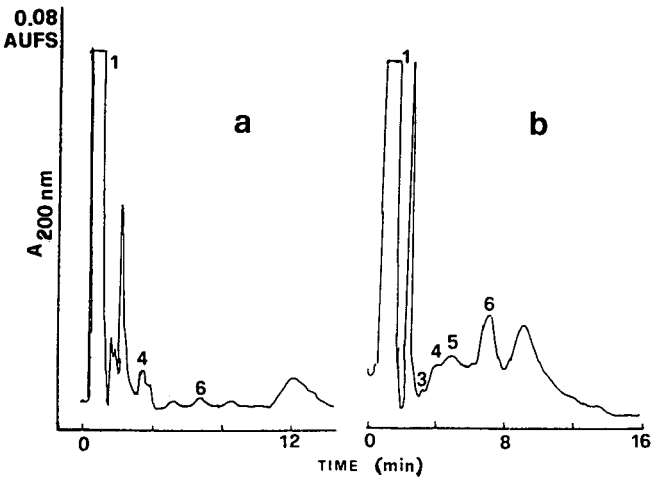

Fig. 3. Examples of HPLC analysis of almond cultivars: (a) *tuono* c.v.; (b) *ferraduel* c.v.

for folic acid to 85.7% for pyridoxine. Furthermore, lower recoveries were associated with a larger variability of data.

Fig. 3 reports examples of analysis after purification of the digest from other two almond cultivars.

As to quantitative data, niacin varied from 1.47 mg per 100 g (*tuono* c.v.) to 3.41 mg per 100 g (*bottara* c.v.) and this range of concentrations agrees with reference data. As for biotin, the range was from 0.12 mg per 100 g (*tuono* c.v. and *scummissa* c.v.) to 0.9 mg per 100 g (*texas* c.v.) and was slightly higher than data in the literature.

Folic acid was present in all the cultivars analyzed, only five cultivars out of nine contained riboflavin (*Fillipo ceo*, *San contino*, *ferraduel*, *scummissa* and *catanese*), and two out of nine contained thiamine (*F. ceo* and *ferraduel*).

Furthermore, it was not possible to detect pyridoxine because of the presence of an interfering peak which was not eliminated with this purification procedure, but this does not mean that it is absent.

For thiamine it should be necessary to enhance detection sensitivity using derivatization to thiochrome and fluorescence detection, as UV detection is not sufficient for the quantitation of this vitamin in almonds.

For folic acid and riboflavin, the resolution is so low as to impair valid integration of the peaks.

Further studies both on the clean-up and concentration steps in sample preparation, and on the chromatographic separation adopting a slightly different mobile phase (*i.e.* a gradient elution) could improve this analysis. For routine purpose therefore this method can be helpful in the analysis of biotin and niacin in almonds.

ACKNOWLEDGEMENT

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CHROMSYMP. 2106

Determination of the ultraviolet absorbance and radioactivity of purine compounds separated by high-performance liquid chromatography

Application to metabolic flux rate analysis

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ABSTRACT

A double detection system for the determination of adenine metabolism in biological tissues using isocratic ion-pair reversed-phase chromatography is presented. Two isocratic ion-pair separations were used: (i) 10 m*M* $NH_4H_2PO_4$, 2 m*M* tetrabutylammonium phosphate (PIC reagent A) and 18% acetonitrile for the determination of nucleotides and (ii) 50 m*M* KH_2PO_4, 1 m*M* PIC reagent A and 1% acetonitrile for the determination of monophosphorylated nucleotides, nucleosides and nucleobases. The parallel detection of ultraviolet absorbance at 254 nm and the radioactivity of separated purine compounds allows the detection of pool sizes and of the specific radioactivities in tracer kinetic experiments. The high-performance liquid chromatography methods were applied to the determination of flux rates during adenine nucleotide metabolism in suspensions of Ehrlich mouse ascites tumour cells. The pathways of adenine metabolism in cells during the proliferation and plateau phases of tumour growth were compared.

INTRODUCTION

Two different phases of growth of Ehrlich ascites tumour in the host can be distinguished: the proliferation or logarithmic phase and the stationary or plateau phase [1,2]. Cells in both growth phases show tremendous differences in the pattern of purines and the uptake of nucleotide precursors [2,3]. It is suggested that the transition of tumour cells from one growth phase into another is related to changes in the turnover rates of the pools of nucleotides, nucleosides and nucleobases. For this reason, the estimation of flux rates in tumour cells of the two growth phases is of biological importance. There are two general methods used for the measurement of metabolic flux rates: the first is based on the determination of concentrations in inhibitory experiments (*e.g.*, using modified nucleosides or nucleobases [4,5]), and the

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second is based on the determination of concentrations and the specific radioactivities in tracer experiments with radioactively labelled purine nucleotide precursors.

Many techniques have been developed for the chromatographic determination of the pool sizes of purine compounds [6–9]. The most common separation systems are ion-pair reversed-phase high-performance liquid chromatography (HPLC) for the separation of nucleotides or the full spectrum of purines [9–13] and reversed-phase HPLC for the determination of nucleosides and nucleobases [14,15]. Two strategies are used for the determination of purine compounds: (i) single-run gradient ion-pair reversed-phase HPLC [9,10] or (ii) the combination of short isocratic separation techniques for groups of metabolites [15,16].

For the assessment of metabolic pathways using tracer kinetics, *e.g.*, for purine metabolism, anion-exchange techniques for the separation of radioactively labelled nucleotides have been used in combination with the measurement of radioactivity in the individual fractions, which are collected, following HPLC [17,18]. The use of on-line detection of radioactivity of purine metabolites would facilitate this analytical procedure, and the aim of this study was to modify and apply two isocratic ion-pair separation techniques for the nucleotides, nucleosides and nucleobases of adenine metabolism for the parallel measurement of pool sizes and radioactivities without fractionation.

EXPERIMENTAL

Materials

Purine reference standards for HPLC were purchased from Boehringer (Mannheim, Germany). The reagents $NH_4H_2PO_4$ and KH_2PO_4 (HPLC grade) were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Acetonitrile and methanol (HPLC grade) were from Merck (Darmstadt, Germany). Tetrabutylammonium phosphate (PIC reagent A) was from Waters Assoc. (Milford, MA, U.S.A.). As a precursor of the flux rate experiments, [U-^{14}C]adenine from Amersham (Little Calfont, U.K.) was used (4 MBq/ml, 10.3 GBq/mmol). Further radioactively labelled compounds which were necessary as standards for the HPLC separations were also obtained from Amersham: [U-^{14}C]ATP (21.3 MBq/mmol), [U-^{14}C]ADP (20.1 MBq/mmol), [U-^{14}C]AMP (1.95 MBq/mmol), [U-^{14}C]GTP (16.9 GBq/mmol), [8-^{14}C]IMP (1.89 GBq/mmol), [U-^{14}C]Ado (18.6 GBq/mmol), [8-^{14}C]Ino (1.96 GBq/mmol), [8-^{14}C]Hyp (2.1 GBq/mmol), [6-^{14}C]Xan and [2-^{14}C]uric acid (1.87 GBq/mmol).

Cell line

Female mice of the ICR strain, weighing approximately 15–20 g, were used. The Ehrlich ascites tumour cells obtained from these animals 7–9 days after inoculation of the tumour were suspended in saline solution at a concentration of $5 \cdot 10^7$ cells per ml; 0.5 ml of this suspension were inoculated into the peritoneal cavity of a healthy animal to propagate the tumour.

Cell preparation

The animals were grouped in the following way: group I, the exponential phase, contained mice on the fifth day after tumour inoculation; group II, the stationary phase, contained mice on the twelfth day after tumour inoculation. The ascitic fluid

was aspirated and Ehrlich mouse ascites cells were washed with a cold (4°C) isotonic saline solution and incubated with continuous stirring at 37°C in a modified Eagle–Borsook solution containing glucose and the proteinogenic amino acids. The cytocrite was maintained at 2%. After a preincubation period of approximately 5 min, radiolabelled adenine was added to the cell suspension (1 μCi/ml of suspension at time zero). After 0.5, 1, 5, 10, 20, 30, 45, 60, 90 and 120 min, aliquots of 0.5 ml of suspension were taken and immediately prepared for HPLC analysis.

HPLC procedures

Sample preparation for HPLC. Aliquots of cell suspensions (0.5 ml) were added to the same volume of ice-cold 6% perchloric acid with stirring. The extracts were centrifuged for 10 min at 1200*g*. The supernatant was neutralized with 1.3 *M* potassium carbonate and centrifuged. The final supernatant was stored at −20°C. After thawing, 50 μl of the supernatant were determined by HPLC.

HPLC equipment. A Perkin Elmer (Norwalk, CT, U.S.A.) system consisting of a 410 pump system, an LC-95 variable-wavelength detector (adjusted to 254 nm), an LCI-100 integrator and a Rheodyne injector was used. The radioactivity detector was from Berthold (Wildbach, Germany). A 5-μm Nova-Pak C_{18} cartridge (100 × 8 mm I.D.) with an RCM 8 × 10 module from Waters Assoc. was also used.

Analytical schedule

Method A. Nucleotides were determined by an isocratic ion-pair reversed-phase HPLC technique. The buffer contained 10 m*M* $NH_4H_2PO_4$, 2 m*M* PIC reagent A and 18% acetonitrile. The flow-rate was 1.5 ml/min.

Method B. Monophosphorylated nucleosides, nucleosides and nucleobases were determined by an isocratic ion-pair reversed-phase technique. The eluent was a buffer containing 50 m*M* KH_2PO_4 (pH 5.1), 1 m*M* PIC reagent A and 1% acetonitrile. The flow-rate was 1.5 ml/min. Peak identification was performed by coelution of the extract with standard mixtures. Therefore, some of the biological samples were divided. One aliquot was analyzed without addition of standard compounds, and to other aliquots reference compounds were added for comparing HPLC runs.

RESULTS AND DISCUSSION

Two HPLC separation systems were used. Both systems were isocratic and they had the advantage of an identical quenching effect of the elution buffer during one run, which is important for the precise measurement of radioactivity.

The first method presented in this paper allows the determination of the mono-, di- and triphosphorylated adenine and guanine nucleosides. As described previously, this procedure does not allow the separation of IMP and GMP [15]. Deoxypurine compounds were excluded prior to this study. Fig. 1 shows chromatograms of the separation of nucleotides (Fig. 1a, measurement of pool sizes; Fig. 1b, measurement of radioactivity). The decreased concentration of acetonitrile (18%) and the lower flow-rate (1.5 ml/min) compared with the separation method reported by Grune *et al.* [15] (20% acetonitrile, 2 ml/min) increase the separation time and improve the detection of radiolabelled nucleotides in the 400-μl flow cell of the radioactivity detector.

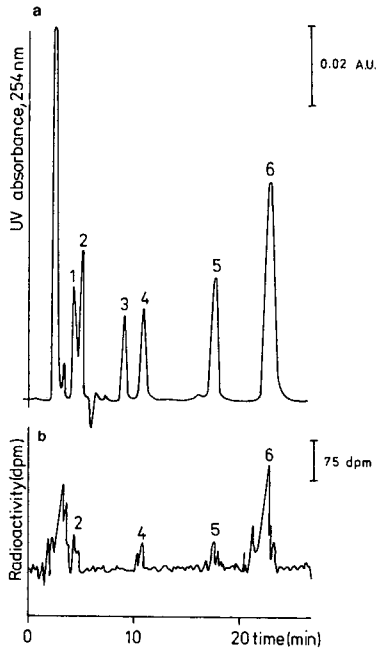

Fig. 1. Chromatograms of separations of nucleotides in extracts of Ehrlich mouse ascites cell suspensions. (a) Measurement of pool sizes; (b) detection of radioactivity. Injection volume, 50 μl; column, Nova-Pak C_{18} cartridge (100 × 8 mm, 5 μm particle size); mobile phase, 10 m*M* $NH_4H_2PO_4$, 2 m*M* PIC reagent A, 18% acetonitrile; flow-rate, 1.5 ml/min. Peaks: 1 = IMP + GMP; 2 = AMP; 3 = GDP; 4 = ADP; 5 = GTP; 6 = ATP.

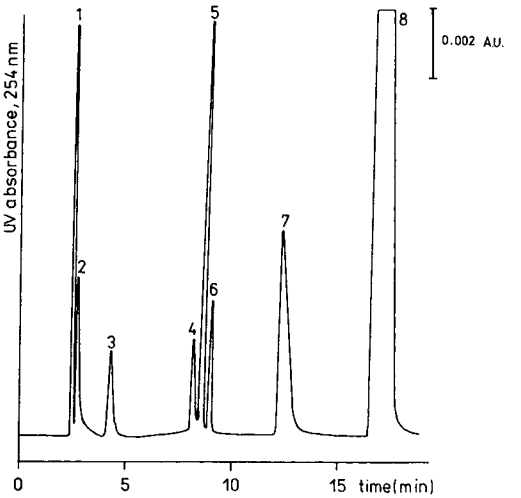

Fig. 2. UV absorbance detection of nucleotide degradation products using an isocratic ion-pair reversed-phase HPLC. The concentration of nucleoside and nucleobase standards was in the range 5–15 μ*M* and the concentration of AMP (peak 8) was 0.5 m*M*. Injection volume, 50 μl; column, Nova-Pak C_{18} cartridge (100 × 8 mm, 5 μm particle size); mobile phase, 50 m*M* KH_2PO_4 (pH 5.1), 1 m*M* PIC reagent A, 1% acetonitrile; flow-rate, 1.5 ml/min. Peaks: 1 = Hyp; 2 = Xan; 3 = uric acid; 4 = Ade; 5 = Ino; 6 = Guo; 7 = Ado; 8 = AMP.

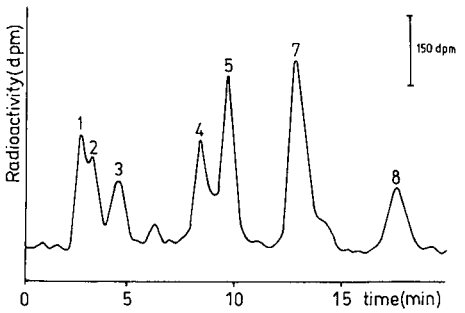

Fig. 3. Determination of radioactivity of authentic radioactive labelled standards of nucleotide degradation products. For chromatographic conditions and peak numbers, see Fig. 2.

The determination of the concentrations and radioactivities of the nucleotide degradation products was performed by an isocratic ion-pair reversed-phase separation method, as described by Togusov *et al.* [16]. However, the eluent in this instance contains a higher concentration of phosphate buffer and only 1% acetonitrile. This method allows the detection of nucleobases, nucleosides and monophosphorylated nucleosides, as shown in Figs. 2 (UV absorbance) and 3 (radioactivity). The difficulty of determining nucleoside levels by reversed-phase HPLC (a decreasing baseline from the front peak of the nucleotides) can be avoided using this method. The nucleotides were either separated very late, or, for example the triphosphorylated nucleosides, were retained at least partially on the column. Therefore, after about fifteen separations the column needs to be rinsed with 20% acetonitrile for 10 min (flow-rate 1.5 ml/min) to remove the remaining nucleosides.

Table I shows the concentrations of purine compounds and Table II the specific

TABLE I

CONCENTRATIONS OF PURINE COMPOUNDS IN SUSPENDED EHRLICH MOUSE ASCITES CELL SUSPENSIONS

Values are given as mean ± S.D. (n = 4).

Purine compound	Concentration (mmol/l cells)	
	Exponential phase	Stationary phase
ATP	4.61 ± 0.23	2.68 ± 0.19
ADP	1.55 ± 0.17	1.09 ± 0.16
AMP	0.65 ± 0.12	0.51 ± 0.10
GTP	0.95 ± 0.23	0.35 ± 0.21
IMP + GMP	0.31 ± 0.07	0.15 ± 0.06
	Concentration (μmol/l cell suspension)	
	Exponential phase	Stationary phase
Ado	0.45 ± 0.11	0.59 ± 0.17
Ade	0.98 ± 0.27	1.44 ± 0.22
Hyp	1.55 ± 0.26	1.44 ± 0.33
Uric acid	3.37 ± 0.29	5.47 ± 0.35

TABLE II

SPECIFIC RADIOACTIVITIES OF PURINE COMPOUNDS OF EHRLICH MOUSE ASCITES CELLS OF DIFFERENT GROWTH PHASES

[^{14}C]Adenine was added to the suspension and 30 min after incubation samples were taken for analysis. Values given are means (n = 4); dpm/pool is the radioactivity of metabolites calculated for the suspension volume of 1 ml, despite the distribution of the metabolites either only inside (ATP, ADP, AMP, GTP, GMP, IMP) or inside and outside (Ado, Ade, Hyp, uric acid) the tumour cells. N.D. = not detected.

Purine compound	Exponential phase		Stationary phase	
	dpm/nmol	dpm/pool	dpm/nmol	dpm/pool
ATP	1545	141 477	725	38 971
ADP	915	28 373	545	11 923
AMP	2365	30 746	2410	24 564
GTP	995	63 162	N.D.	N.D.
IMP + GMP	2325	14 407	4030	11 923
Ado	45 509	20 479	24 044	14 186
Ade	485 985	476 265	842 145	1 212 688
Hyp	18 946	29 366	6900	9936
Uric acid	6289	21 197	2522	13 795
Nucleic acids and others	–	1 394 528	–	882 014
Total	–	2 220 000	–	2 220 000

and total radioactivities of the pools after 30 min of incubation of the Ehrlich ascites tumour cells in the presence of ^{14}C-labelled adenine. The growth-dependent changes in the concentration of purine compounds reported previously [2] are confirmed. The distribution of the radiolabelled purine precursors shows a higher radioactivity in most components of purine metabolism in the cells during the proliferating phase of tumour growth, suggesting higher enzyme activities and metabolic flux rates in these cells compared with those of the plateau phase.

The methods presented in this paper allow the determination of adenine nucleotides and their degradation products, in addition to the determination of their ^{14}C-labelling, without fractionation of the eluent. It is therefore possible to calculate metabolic flux rates in the pathways of adenine metabolism.

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CHROMSYMP. 2126

Hypertensive congenital adrenal enzymatic defects detected by high-performance liquid chromatography of corticosteroids

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ABSTRACT

The simultaneous measurement of the adrenal deoxycorticosterone (DOC), 18-OH-DOC, corticosterone (B), 18-OH-B, 11-deoxycortisol (S) and cortisol (F) present in human plasma in cases of adrenal dysfunction was accomplished using a high-performance liquid chromatographic (HPLC) system with a UV detector and with a radioimmunoassay (RIA). After a solid-phase extraction, plasma samples were separated by HPLC using a gradient of water–acetonitrile–ethanol on a radial compressed reversed-phase column. In a 70-min cycle, a complete separation of adrenal steroids was accomplished. The UV detector allowed direct measurement of F in each plasma sample while in selected cases B and S were directly determined. It was therefore possible quickly to identify patients with hypertensive congenital adrenal enzymatic defects with this method: the 17-α-hydroxylase deficiency characterized by the absence of measurable levels of F with an evident peak corresponding to B and the 11-β-hydroxylase deficiency in which high levels of S without F are detected. The RIA of DOC, B, 18-OH-DOC and 18-OH-B complete the characterization of the adrenal defect. Therefore, with this HPLC method it is possible to recognize the major hypertensive adrenal enzymatic deficiencies such as the defect of 17-α-hydroxylase or 11-β-hydroxylase. With "RIA" detectors an almost complete spectrum of adrenal steroid secretion can be obtained.

INTRODUCTION

The investigation of adrenal enzymatic defects requires the specific determination of all major intermediates and end products of adrenal steroidogenesis. High-performance liquid chromatography (HPLC) offers many advantages over older types of chromatography such as higher resolution, a higher degree of reproducibility and, as has been proposed in recent years, the possibility of full automation [1,2] for the separation of a number of corticosteroids [3–5]. Therefore, it has been applied to identify and determine different hormones such as corticosteroids, glucocorticoids, estrogens and a variety of "non-classic" or so-called "minor" steroids and steroid conjugates in several conditions in plasma, urine and various tissue samples.

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An efficient separation and simultaneous measurement of corticosteroids in human plasma, particularly those of early step steroids, were recently achieved by reversed-phase HPLC and radioimmunoassay (RIA) [6]. In this paper, we describe the usefulness of this HPLC method as a diagnostic tool for patients with adrenal enzymatic defect.

EXPERIMENTAL

The HPLC–RIA determination of the adrenal steroids was performed as described previously [6]. Briefly, after the solid-phase extraction of plasma with Chem-Elut cartridges using methylene chloride as solvent, the samples were separated by HPLC using a ternary gradient (water–acetonitrile–ethanol) on a radial compressed reversed-phase column. The recovery was calculated by adding cortisone (E) and testosterone as internal standards and a tritiated aliquot of each steroid was analysed.

The sensitivity of the on-line spectrophotometer allows the direct assay of the cortisol (F) concentration and also, in some instances, that of corticosterone (B) and 11-deoxycortisol (S). For the other steroids, the selected fractions were collected, extracted and aliquots submitted to RIA/ or to recovery counting. RIA of the single steroids were performed with tritiated tracers and antibodies kindly provided by Professor P. Vecsei (Heidelberg, Germany) [7].

RESULTS

We studied six patients, phenotypically female (among which three were sisters), with 17-α-hydroxylase deficiency; when the diagnosis was made, they presented as young adult females with primary amenorrhea, hypertension and hypokalaemia. The two brothers, affected by 11-β-hydroxylase deficiency, presented hypertension, mild hypokalaemia and pseudoprecocious puberty.

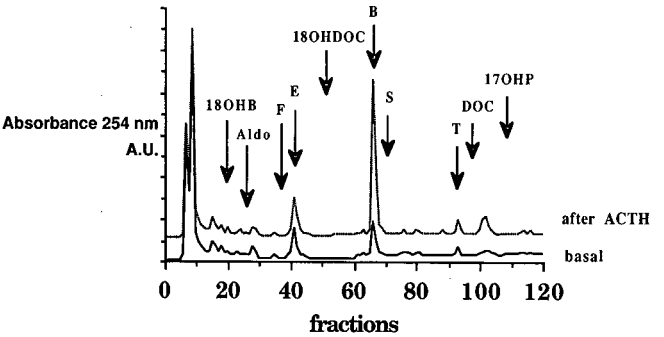

Fig. 1. Chromatograms of a plasma sample from a patient affected by 17-OHDS before and after ACTH administration. The arrow indicates the retention times of the steroids. Cortisone (E) and testosterone (T) are internal standards. Each fraction is 25 s. Column, RP C_{18} (4 μm), radial compressed. Solvent A, acetonitrile–ethanol (99:1); solvent B, water–ethanol (99:1); gradient profile, three linear steps from 28% to 44% of solvent A.

The concentrations of F, B and S were determined using the ratio of the peak area for the steroid to that of 1.14 μmol/l of cortisone (E) at a wavelength of 254 nm. The detection limit was 0.02 μmol/l. In the patients with 17-α-hydroxylase deficiency syndrome (17-OHDS) and 11-β-hydroxylase deficiency syndrome (11-OHDS) F is undetectable whereas B in the first case or S in the latter is very high, being more than ten times the upper limit of the normal range.

In all the patients the concentrations of B, deoxycorticosterone (DOC), 18-OH-DOC and 18-OH-B were also measured after off-line single-steroid RIA. In the 17-OHDS case, B, DOC, 18-OH-DOC and 18-OH-B are very high whereas F is very low. The cases of 11-OHDS have high DOC concentrations and reduced levels of F, B, 18-OH-DOC and 18-OH-B.

DISCUSSION

The adrenal gland plays a prominent role in the regulation of blood pressure. The hypersecretion of the most potent mineralocorticoid hormone, aldosterone, is responsible for the syndrome of primary aldosteronism which is characterized by hypertension, suppression of the renin–angiotensin system and hypokalaemia. Less frequently, adrenal hypertension is produced by other mineralocorticoid hormones (MCH) as in the DOC-secreting form of congenital adrenal hyperplasia, 11-β- and 17-α-hydroxylase deficiency [8,9]. In cases of 11-OHDS, the enzymatic system is missing in the mineralocorticoid and in the glucocorticoid pathways of the adrenal cortex, leading to impaired production of F and B. As a consequence, patients with 11-OHDS have high ACTH levels with high concentrations of all steroids proximal to the defect, *i.e.*, progesterone, 17-OH-progesterone, androgens, DOC and S. In cases of 17-OHDS, all steroids requiring hydroxylation in position 17 (glucocorticoids, androgens and estrogens) are very low whereas the MCH pathway, which does not require 17-α-hydroxylation, is stimulated by the excessive ACTH drive, due to the absence of F; excessive amounts of DOC, B, 18-OH-DOC and 18-OH-B are produced.

In the plasma of patients affected by adrenal enzymatic defects, with the "on-line" photometer, B or S was directly measured, whereas the peak of F, which is easily detectable in normal samples, was almost absent. Using two key steroids, F and B or F and S, the 17-OHDS and 11-OHDS cases are immediately identified. This assay seems to be very specific as this HPLC method allows a complete separation of the adrenal steroids (see the retention times of standards in Fig. 1) and the mean relative standard deviation (R.S.D.) of the retention times of individual steroids is very low, ranging between 1.9 and 0.8%; the intra-assay R.S.D.s for S, B and F are 8.1, 8.7 and 8.0%, respectively. The assay is rapid, requiring a few hours for twelve samples, as only the first two steps of the entire procedure are needed, namely extraction and HPLC separation; moreover, only a limited volume of plasma (1 ml) is required.

The concentrations of 18-OH-B, DOC and 18-OH-DOC are below the sensitivity of the photometer and cannot be measured by this method. Obviously, with the addition of the post-HPLC off-line single-steroid RIA this can be achieved and a more complete biochemical characterization can be obtained. This is required for a more sophisticated evaluation of patients. Nevertheless, when a rapid and specific diagnostic tool is needed, HPLC with direct assay of F and B or S is useful. Additional diagnostic information can be achieved after ACTH stimulation; in fact, as in the

example of 17-OHDS in Fig. 1, ACTH greatly stimulate B secretion (before the 17-α-hydroxylase) but failed to stimulate F (after the 17-α-hydroxylase) with a magnification of the enzymatic block.

In conclusion, this HPLC method can be used as a diagnostic tool in cases of 17-OHDS and 11-OHDS; even if limited to the direct assay of F, B and S, a biochemical pattern suggestive of an adrenal enzymatic defect can be obtained. Owing to its specificity and simplicity, this method can be proposed for screening programmes or large-scale assays.

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CHROMSYMP. 2119

Purine and pyrimidine compounds in murine peritoneal macrophages cultured *in vitro*

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ABSTRACT

Extracts of murine peritoneal macrophages were analysed by ion-pair reversed-phase high-performance liquid chromatography during incubation at 37°C *in vitro*. Four-step gradient elution was applied to an ODS column (250 × 4.6 mm I.D.) at a flow-rate of 1.3 ml/min, allowing the separation of hypoxanthine, inosine, guanosine, adenosine, IMP, CDP, AMP, GDP, UDP, ADP, CTP, GTP, UTP and ATP within 50 min. Samples of $0.4 \cdot 10^6$–$0.5 \cdot 10^6$ cells were washed twice with RPMI 1640 medium and extracted with perchloric acid. Nucleotide concentrations of murine peritoneal macrophages did not change during incubation for 4 days *in vitro*.

INTRODUCTION

Purine and pyrimidine compounds are of major importance for a multitude of cellular functions. Synthesis and catabolism of nucleotides are well understood and reviewed [1–3]. Extensive metabolic studies have been performed on liver, heart, and red blood cells. However, only a limited number of investigations have been made on nucleotide metabolism in white blood cells, although there are relationships between nucleotide metabolism and several specific functions of these cells, such as the following: phagocytosis is accompaied by a decline in ATP [4]; the activation of lymphocytes is promoted by ATP [5]; guanine nucleotides activate the O_2^--generating NADPH-dependent oxidase [6] and adenosine inhibits O_2^- generation [7]; an ecto-5′-nucleotidase cleaves external AMP to adenosine, which is then transported into the macrophage by a purine nucleoside carrier [8]; and the activity of this enzyme characterizes functionally different cell populations [9]. A genetic defect of adenosine deaminase disturbs lymphocyte proliferation and causes immunodeficiency [10].

A thorough study of the nucleotide profile in lymphocytes and macrophages has not been published. This work was aimed at the determination of a wide range of purine metabolites in macrophages by gradient ion-pair reversed-phase high-per-

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formance liquid chromatography (HPLC), allowing the separation of purine nucleotides, nucleosides and nucleobases in a single run.

EXPERIMENTAL

Macrophage culture

A 2-ml volume of 10% proteose peptone (Difco Labs., Detroit, MI, U.S.A.) was injected into the peritoneal cavities of male mice (age 2 months). After 4 days, 10 ml of RPMI 1640 (Flow Labs., Irvine, U.K.) were injected into the peritoneal cavity and the suspended macrophages were aspirated. The cells were washed twice with that medium. Then $10 \cdot 10^6$ cells were suspended in RPMI 1640 containing 10% foetal calf serum. The suspension was placed in plastic culture dishes of 35 mm diameter (Terumo, Tokyo, Japan). The cells were allowed to adhere at 37°C under 5% carbon dioxide during a 3-h incubation. The non-adhering cells were removed by rinsing twice with RPMI 1640. The incubation was continued and after 24 h the medium was exchanged [11]. At this time and at the fourth day of incubation aliquots were sampled for nucleotide analysis.

Sample extraction

Macrophages were scraped off, washed twice with RPMI medium and nucleotides were immediately extracted with 6% perchloric acid; after centrifugation, the supernatant was neutralized with potassium hydroxide. The samples were cleaned from insoluble potassium chlorate by centrifugation at 900 *g* for 3 min and in addition by percolation through a 0.2-μm membrane filter (Sartorius, Germany).

HPLC equipment

The HPLC system consisted of equipment from Waters Assoc. (Milford, MA, U.S.A.), with two Model 510 HPLC pumps, an automated gradient controller, a programmable multi-wavelength detector (peak identification by UV absorbance measurement at 254 and 280 nm), a Model 745 data module and Rheodyne Model 7125 injector with a 50-μl sample loop.

Chromatographic conditions

Buffer A [10 m*M* $NH_4H_2PO_4$ (Merck, Darmstadt, Germany) + 2 m*M* tetrabutylammonium phosphate (PIC Reagent A; Waters Assoc.)] and buffer B [buffer A containing 20% (v/v) acetonitrile (LiChrosolv, Merck)] were used. The flow-rate was 1.3 ml/min. A Supelcosil ODS column (250 × 4.6 mm I.D.) was applied.

The following gradient was used: 5 min isocratic with 100% buffer A, followed by a 12-min linear gradient up to 80% B, then 30 min isocratic with 80% B–20% A followed by a 2-min linear gradient to 100% B, returning in 5 min to 100% buffer A.

Quantification and peak identification

In perchloric acid extracts, recoveries of 81 ± 12% for ATP and 76 ± 14% for hypoxanthine were obtained. The recoveries for the other compounds were between these values. Different amounts of each standard compound were injected and the linearity of the calibration graph for the particular concentration range was checked (nucleosides, nucleobases, 50–500 pmol; nucleotides, 200–1500 pmol). Peak identi-

fication was performed by coelution of the macrophage extracts with known standards.

RESULTS AND DISCUSSION

Ion-pair reversed-phase separation

Ion-pair methods were used initially to enhance the selectivity in solvent extraction and later they were introduced to improve reversed-phase LC separations [12]. The ion-pair reversed-phase (IP-RP) mode is applied nowadays to separate bases and acids, pharmaceuticals, amino acids, peptides, proteins and nucleic acids and for chiral separations [12,13]. Nucleotides interact with cationic ion-pair reagents owing to their ionic properties and in this way a good retention of these compounds on C_{18} columns is possible. In the experiments performed here, tetrabutylammonium phosphate was chosen. The pH of the buffers was 5.8. Hypoxanthine, inosine, guanosine, adenosine, IMP, CDP, AMP, GDP, UDP, ADP, CTP, GTP, UTP and ATP eluted as shown in Fig. 1. c-AMP elutes after ATP (not shown in Fig. 1). Similar IP-RP separations of nucleotide standards have been reported [14].

Nucleotide concentrations in murine peritoneal macrophages

Murine peritoneal macrophages were cultivated for 4 days at 37°C. Perchloric acid extracts were analysed by the IP-RP gradient separation described at the first and fourth days. Fig. 2 shows the corresponding elution profile of an extract obtained after

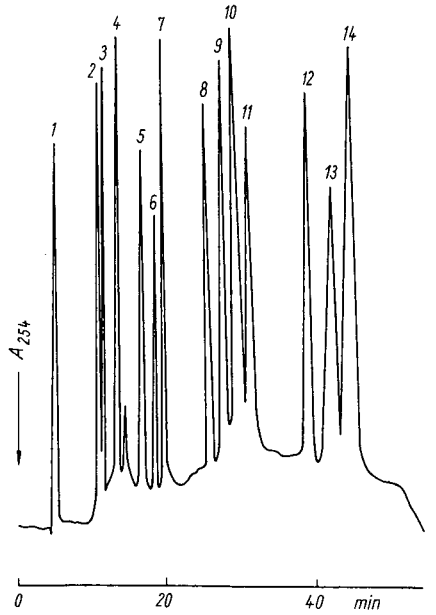

Fig. 1. IP-RP separation of nucleotide, nucleoside and nucleobase standards (400 pmol each). Detection at 254 nm. Column, Supelcosil ODS (250 × 4.6 mm I.D.); flow-rate, 1.3 ml/min. Peaks: 1 = hypoxanthine; 2 = inosine; 3 = guanosine; 4 = adenosine; 5 = IMP; 6 = CDP; 7 = AMP; 8 = GDP; 9 = UDP; 10 = ADP; 11 = CTP; 12 = GTP; 13 = UTP; 14 = ATP.

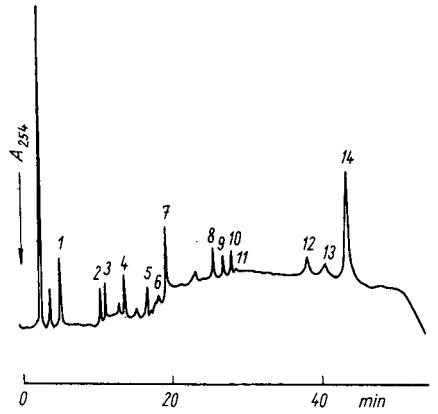

Fig. 2. Chromatographic elution profile of an extract of murine peritoneal macrophages after incubation *in vitro* for 4 days. $0.4 \cdot 10^6$ cells were used for the extraction.

in vitro cultivation for 4 days. Nucleotide concentrations were not significantly changed during incubation (Table I), indicating that the metabolic integrity during incubation was maintained. This is in accordance with data on unstimulated granulocytes cultivated for 3 days [15]. RPMI 1640 is known to be a good storage

TABLE I

NUCLEOTIDE CONCENTRATIONS IN EXTRACTS OF MURINE PERITONEAL MACROPHAGES

Values are given in pmol per 10^6 cells (mean ± S.E.M., $n = 5$). Macrophages harvested from the peritoneal cavity of 2-month-old mice treated with 10% proteose peptone were plated in culture dishes in RPMI 1640 medium with 10% foetal calf serum. Non-adhering cells were removed by rinsing with culture medium after incubation for 24 h. The incubation was performed at 37°C under 5% CO_2. Samples were scraped off from the dishes, washed twice by rinsing with RPMI medium and were immediately extracted with 6% perchloric acid.

Nucleotide	Time of cultivation	
	1 day	4 days
Hypoxanthine	11.3 ± 3.2	13.2 ± 5.4
Inosine	4.3 ± 0.7	5.1 ± 1.2
Guanosine	4.6 ± 0.5	4.9 ± 0.8
Adenosine	2.8 ± 0.9	3.1 ± 1.2
IMP	3.1 ± 1.1	3.0 ± 1.1
CDP	2.5 ± 1.6	2.0 ± 1.5
AMP	22.5 ± 6.5	23.5 ± 7.1
GDP	61 ± 12	62 ± 16
UDP	32 ± 18	33 ± 19
ADP	55 ± 21	51 ± 22
CTP	3.8 ± 0.9	3.0 ± 1.8
GTP	27 ± 9	26 ± 12
UTP	18 ± 8	18 ± 11
ATP	1398 ± 172	1314 ± 235

TABLE II

NUCLEOTIDE CONCENTRATIONS IN LYMPHOMA CELLS, LYMPHOCYTES, POLYMORPHONUCLEAR LEUCOCYTES AND MACROPHAGES

Values are given in pmol per 10^6 cells.

Nucleotide	Cells							
	Lymphoma L5178Y [3]	Lymphoma K. 562 [17]	Lymphocytes			Lymphocytes (man) [19]	Polymorpho nuclear (rat) [20]	Peritoneal macrophage (mouse) (this work)
			Man [18]	Pig [18]	Rat [18]			
ATP	2600	557	855	1107	237	826	830	1398
ADP		73.5	302	475	95		131	55
AMP		54	43	43	70		28	22.5
Ado		10.6					4.3	2.8
GTP	700	169.5	182	205	80	200		27
GDP		50.5	53	67	32			61
Guo								4.6
UTP		126.7	142	96	53	120		18
UDP		12.4	20	15	24			32
IMP		162					46	31
Ino							9	4.3
Hyp		25					21	11.3
CTP			39	34	27	83		3.8
CDP			12	16	11			2.5

medium also for neutrophils [16]. During phagocytosis the ATP concentration decreases and the nucleotide metabolism deteriorates [4].

In Table II, concentrations of ribonucleotides in white blood cells are surveyed.

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Analysis of biomass pyrolysis oils by a combination of various liquid chromatographic techniques and gas chromatography–mass spectrometry

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ABSTRACT

A strategy for the overall analysis of pyrolysis oils is decribed based on the combination of a previously developed fractionation, the improved API 60 fractionation, with high-performance analytical techniques used in series, such as size-exclusion chromatography and capillary gas chromatography–mass spectrometry. As an example, the results obtained for the neutral and acid fractions resulting from the improved API 60 fractionation of pyrolysis oils prepared by carbonization of different species (hornbeam, poplar, pine) are given.

INTRODUCTION

Successive world energy crises and large increases in the prices of oil derivatives have caused renewed interest in the different possibilities offered by the valorization of biomass as an energy source and in the chemical exploitation of wood pyrolysis by-products.

Pyrolysis oils can be considered as a real source of useful chemicals. Although product such as acetic acid or methanol are now more easily obtained from synthetic processes, the situation is different for numerous chemicals extractable from pyrolysis oils. Unfortunately, pyrolysis oils, which are very complex matrices constituted by hundreds of compounds with very different polarities, change with time. This instability is unquestionably a handicap in their exploitation and consequently their valorization. The stabilization of pyrolysis oils requires first the chemical and structural characterization of this complex matrix. Unlike pyroligneous acids, which are well known and have been the subject of many studies [1–5], few structural data are available on tars from biomass. Until recently studies on these organic matrices were

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mainly oriented towards the identification of particular compounds families, *e.g.*, polycyclicaromatic hydrocarbons [6–9], phenols [9–14], light fatty acids [15–18] and carbonyl compounds [19,20].

In 1986 Elliott [21,22], using capillary gas chromatography (GC) and mass spectrometry (MS), characterized for the first time the tars resulting from wood carbonization. This fundamental study constitutes the first overall approach to the analysis of wood carbonization tars as previously only a detailed identification of polyaromatic hydrocarbons had been reported [23]. However, the method used by Elliott to simplify the organic matrix prior to analysis by GC—MS, *viz.*, extraction with dichloromethane, was limited because of the low solubility of highly polar compounds in this solvent. Consequently, the results obtained, although very interesting, give an overestimation of low-polarity compounds to the detriment of high-polarity compounds. In order to avoid these difficulties, we decided to use a set of liquid chromatographic techniques to fractionate the complex organic matrix of pyrolysis oils. We adapted several techniques developed for the analysis of heavy oil residues [24,25], *i.e.*, the coupling of chromatographic and spectroscopic techniques, to obtain information about the structure of pyrolysis oils.

EXPERIMENTAL

Reagents

Cyclohexane, benzene, methanol, acetonitrile and tetrahydrofuran (THF) used for preparative ion-exchange chromatography were obtained from Merck (Darmstadt, Germany) and were purified by distillation. Water was purified by reverse osmosis and filtration using a Milli-RO + Milli-Q system from Millipore (Molsheim, France).

The water content of oils was determined using a Karl Fischer Automat E 547 apparatus (Metrohm, Herisau, Switzerland) with the reagents Re Aquant titrant and Re Aquant solvent (Baker, Deventer, The Netherlands).

Preparative ion-exchange chromatography

Ion-exchange chromatographic fractionations were performed using a preparative system built in our laboratory as described previously [26]. The experimental assembly was a modular component system consisting of a Model MCP 110 pneumatic pump (Haskel, Burbank, CA, U.S.A.), a pulse damper (Touzart et Matignon, Vitry/Seine, France), a splitter (Waters Assoc., Milford, MA, U.S.A.) an injection valve (Rheodyne, Cotati, CA, U.S.A.) equipped with a 10-ml sample loop, a heat exchanger made from a 2.5 m × 1.65 mm I.D. length of stainless-steel tubing shaped in a spiral and fitted in a heating jacket and three three-way valves (Hoke, Cresskill, NJ, U.S.A.) allowing the columns either to be set on-line or to be by-passed. The different parts were connected with 4.7 mm I.D. stainless-steel tubing (Touzart et Matignon), except for the splitter–detector connection, where 0.25 mm I.D. stainless-steel tubing was used. The two columns utilized were two 30 cm × 2 cm I.D. sections of stainless-steel tubing one dry packed with 15–60-mesh Amberlite IRA 904 and the other with 16–50-mesh Amberlyst A15 (Rohm and Haas, Philadelphia, PA, U.S.A.).

In order to exhibit maximum effectiveness for pyrolysis oil applications, the resins must be converted to the hydroxide form (IRA 904) and hydrogen form (A15)

TABLE I

PREPARATION OF AND CONDITIONING PROCEDURE FOR ION-EXCHANGE RESINS

Resin	Washing	Neutralization	Activation	Neutralization	Conditioning for 8 h: solvent
A 15	CH_3OH–KOH (90:10)	Distilled water	CH_3OH–HCl (90:10)	Distilled water	CH_3OH, CH_3CN, benzene, cyclohexane
IRA 904	CH_3OH–HCl (90:10)	Distilled water	CH_3OH–KOH (90:10)	Distilled water	CH_3OH, THF, benzene, cyclohexane

according to a method derived from that recommended by Jewell [27] (Table I). Column I was packed with the anion-exchange resin and column II with the cation-exchange resin. Through control by two valves the circulating fluid was pumped through either one or both columns.

The sample (3.1 g) was dissolved using ultrasonic dispersion in the minimum volume of benzene–cyclohexane (75:25) and the solution was then fed into the sample loop injector. It must be noted that with fast pyrolysis oil, 17% of the sample is insoluble in this mixture, in contrast to the other samples, which are completely soluble. The elution sequence and the acid–base characteristics of the fractions collected are reported in Table II.

Size-exclusion chromatography

The apparatus consisted of a Model 6000 A dual-piston pump, a U6K universal injector, a model M 440 UV–visible detector and a R 401 differential refractometric detector (all from Waters Assoc.). The signals from the detectors were displayed on a Kipp & Zonen (Delft, The Netherlands) recorder.

The chromatographic separations were performed on two columns, a 5-μm μSpherogel 50 Å (300 mm × 7.7 mm I.D.) (Beckman, Fullerton, CA, U.S.A.) coupled with a 7-μm μStyragel 100 Å (300 mm × 7.7 mm I.D.) (Waters Assoc.). The flow-rate of the mobile phase (THF) was 0.6 ml/min.

GC analyses and GC–MS coupling

GC analyses were performed using a Hewlett-Packard Model 5880 A gas chromatograph fitted with a 25-m fused-silica column coated with CP Sil 5 (Chrompack, Middelburg, The Netherlands) and a flame ionization detector. The conditions used were as follows: carrier gas, nitrogen U (Air Liquide, St-Quentin en Yvelines, France), inlet pressure 0.9 bar; injector temperature, 275°C; detector temperature, 280°C; splitting ratio, 1:30; injection volume, 1 μl of a 10 mg/ml solution; temperature programme, isothermal at 40°C for 5 min, linear gradient at 3°C/min to 150°C, then at 4°C/min to 250°C, isothermal at 250°C for 30 min.

For GC–MS, a model R 10-10 quadrupole spectrometer (Delsi Nermag Instruments, Argenteuil, France) fitted with the same column as above was used. The chromatographic conditions were as follows: carrier gas, Helium U (Air Liquide),

inlet pressure 0.7 bar; injector temperature, 240°C; interface temperature, 285°C; splitless injection, 10 s; injection volume, 2.5 μl of a 10 mg/ml solution. The mass spectrometric conditions analysis were as follows: repeller potential, 6.6 V; source potential, 6 V; filament current intensity, 180 mA; electron energy, 70 eV; mass range, 31–450 u.

Spectra were interpreted by matching the results with library spectra and with known fragmentation patterns obtained from standard compounds. These standard compounds are marked with asterisks in Tables IV–VI.

Samples

The pyroligneous oils were derived from the carbonization of different wood species (hornbeam, pine and poplar). The logs were dried in a drying stove at 105°C, then stored for 1 month under ambient conditions. The wood dryness was then 92–94%. The carbonization was performed at the Centre Technique Forestier Tropical, using a Herman-Moritz oven, using the following programme: for slow pyrolysis, linear temperature gradient from 20 to 110°C in 3 h, isothermal at 110°C for 18 h (drying), linear temperature gradient from 100 to 500°C in 5 h, isothermal at 500°C for 5 h (cooking), cooling for 20 h and for fast pyrolysis, linear temperature gradient from 20 to 500°C in 2 h, isothermal at 500°C for 1 h (cooking), cooling for 20 h.

The characteristics of the pyroligneous oils were as follows: hornbeam (slow pyrolysis), 49.3 g of oil from 100 g of dry wood, including 7.9 g of tar, specific gravity 1.18 g/ml; hornbeam (fast pyrolysis), 54.1 g of oil from 100 g of dry wood, including 8.5 g of tar, specific gravity 1.07 g/ml; pine (slow pyrolysis), 44.9 g of oil from 100 g of dry wood, including 10.5 g of tar, specific gravity 1.04 g/ml; poplar (slow pyrolysis), 36.8 g of oil from 100 g of dry wood, including 6 g of tar, specific gravity 1.07 g/ml.

RESULTS AND DISCUSSION

The strategy developed combines two liquid-phase chromatographic techniques (ion-exchange and size-exclusion chromatography) with coupled GC–MS. The pyrolysis oils (after thorough azeotropic dehydration) were submitted first to a functional sorting according to a technique derived from the API 60 procedure [28]. This method uses the differences in interactions in an organic medium between a solute and ion-exchange resins (styrene–divinylbenzene copolymers, bonded to sulphonates and ammonium moieties in cation and anion exchangers respectively). As the mobile phase is organic, there is strictly no ion exchange but rather an association between functional groups in the sample molecules and functional groups of the resins constituting the stationary phase. This association is more or less strong depending on the acid–base characteristics of the sample components.

The sample is deposited on the resin surface by means of a solvent (the least polar possible) compatible with its solubility. Bonds of different strengths are formed and are then progressively destroyed as a function of their nature by thorough elution using mobile phases of increasing polarity. Hence the components are eluted according to their increasing acid–base characteristics.

Originally, this chromatographic technique was long and tedious. Some years ago, it was improved in our laboratory [26] by means of the device described under Experimental. We can perform a complete acid–base separation in 7 h compared with

TABLE II

ELUTION SEQUENCE AND ACID–BASE CHARACTERISTICS OF THE FRACTIONS COLLECTED IN PYROLYSIS OIL ANALYSIS

Ion exchanger	Eluent	Acid–base characteristic of the fraction collected
A15 + IRA 904 (coupled)	Benzene–cyclohexane (75:25)	Neutral
IRA 904	Benzene	Weak acids
	Benzene–acetonitrile	Medium acids
	Acetonitrile	Strong acids
	Methanol	Very strong acids
A15	Benzene	Weak bases
	Benzene–THF	Medium bases
	THF	Strong bases
	Methanol	Very strong bases

more than 8 days using the original method, obtaining nine fractions with different acid–base characteristics.

As the improved API 60 method was developed for the analysis of heavy oil fractions, an adaptation was necessary for application to pyrolysis oils. Some of the solvents used in the separation of heavy oil fractions, such as acetic acid and diethylamine, are not suitable for the analysis of wood pyrolysis oils. These solvents gave solvates which were too stable. The elution sequence used to analyse pyrolysis oils and the acid–base characteristics of the fractions collected are reported in Table II.

The acid–base balances obtained after analysis of various species are reported in Table III. Yields were calculated with respect to the mass of dry tar injected. The main features of the results are as follows. The partitioning of acid–base fractions depends on the nature of both the wood species and the process (fast or slow pyrolysis). With hornbeam and poplar a similar acid–base balance is obtained, but the results are different for pine (softwood) pyrolysis. However, although mass balances

TABLE III

ACID–BASE REPARTITION OF PYROLYSIS OILS AS A FUNCTION OF WOOD SPECIES (MASS BALANCE)

Wood species	Acid–base characteristic of fractions collected			
	Neutral (%)	Acidic (%)	Basic (%)	Total yield (%)[a]
Hornbeam[b]	37	33.5	28	98.5
Pine[b]	65	16.5	13.5	95
Poplar[b]	35	29	27	91
Hornbeam[c]	54	22	18.5	94.5

[a] Calculated with respect to the dry tar mass injected before dissolution.
[b] Slow pyrolysis.
[c] Fast pyrolysis.

of poplar and hornbeam are similar, their qualitative compositions are different, as shown by the capillary gas chromatograms of the various acid–base fractions of their pyrolysis oils (see Fig. 1). The structures of the compounds attributed to the main peaks are given. The attributions made done by comparison of the chromatograms of neutral and acidic fractions and of GC–MS analyses of their neutral and acidic subfractions obtained after size-exclusion chromatography. With the basic fraction, the subfractions obtained after size-exclusion chromatography were still too complex to allow GC–MS analyses and no attribution was attempted. Totally different balances were obtained with the same species (here hornbeam) as a function of pyrolysis conditions (slow or fast). Finally apart from the neutral fraction, which is the most abundant regardless of wood species, the medium-acid fraction and the medium-basic fraction are predominant in acids and bases.

After this first separation, the organic matrix is fairly simplified, but the complexity of the fractions collected remains too great and impedes identification of the components by GC–MS. Therefore we applied a second preparative chromatographic step to the acid–base fractions collected. This second step is based on size-exclusion chromatography.

Size-exclusion chromatography has been applied in the field of wood liquefaction [29–31]. For instance, Shen *et al.* [32] performed such a separation and four

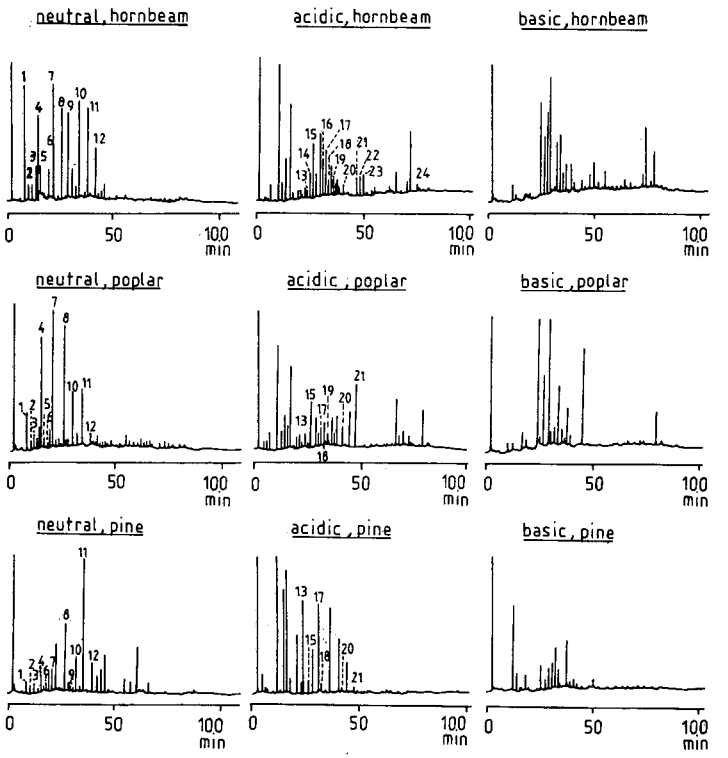

Fig. 1. Capillary gas chromatograms of various acid–base fractions of pyrolysis oils obtained by slow pyrolysis.

classes of components were separated from a pyrolysis oil: heavy hydrocarbons, light hydrocarbons and esters, phenolic derivatives and aromatic compounds.

We applied this technique of sorting as a function of molecular size of each of the fractions obtained from the API 60 separation. We resolved each of the acid–base fractions into three subfractions with different molecular weights. As an example, Fig. 2 shows the separations of the predominant acid–base fractions for hornbeam wood with slow pyrolysis. We checked that for all three subfractions obtained from each acid–base fraction, the recovery yields were quantitative within the experimental precision.

After this second preparative separation, we obtained 27 fractions for each of the pyrolysis oils analysed and we attempted to identify the components by capillary GC–MS. Every fraction was studied using two ionization modes: electron impact at 70 eV and chemical ionization by ammonia and methane. These two ionization modes are complementary: the former allows the fragmentation of molecules and gives important structural information and the latter is used to obtain molecular weights.

For each fraction, a comparision is made between the total ion current in the electron impact and chemical ionization modes. The molecular weight is then obtained for each peak on the gas chromatogram. After the determination of molecular weights, we searched for the ten best correlations between the electron impact mass spectra and the mass spectral library. Thus, we obtained the structures of a number of fragments and hypothetical structures were established. Among these hypothetical structures, the most likely according to the physico-chemical properties of the compound (acid–base characteristics, polarity and chromatographic behaviour) was retained.

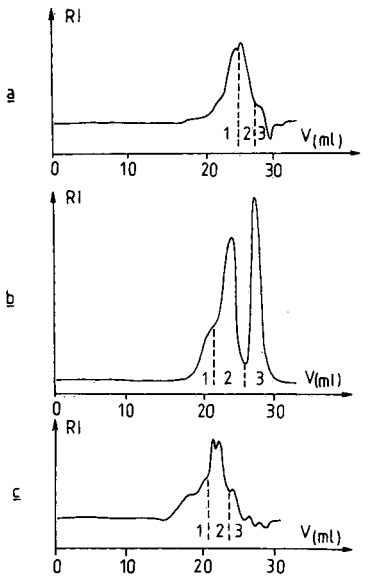

Fig. 2. Preparative high-performance size-exclusion chromatography of acid–base fractions from pyrolysis oils of hornbeam wood obtained by slow pyrolysis: (a) neutral fraction; (b) medium acid fraction: (c) medium basic fraction. V = Retention volume; RI = refractive index.

TABLE IV

CONSTITUENTS OF THE NEUTRAL FRACTION OF A HORNBEAM PYROLYSIS OIL OBTAINED BY SLOW PYROLYSIS

Compound[a]	Peak No.[b]	Compound[a]	Peak No.[b]
Esters, ketones and aldehydes		*Aromatics*	
Methylfuran carboxylate		Dimethylethyl benzene	
2-Butanone acetate		Diethyl benzene	1
Tetrahydrofuranyl acetate		Tetramethylbenzene	2
Propyl acetate		Ethylpropylbenzene	
Acetylcyclopentene		3-Phenyl-1-butene	3
Acetylcyclohexene		Methylindene	
Ethyl methylbenzoate		Ethylstyrene	
Methyl dimethoxybenzoate		Propylstyrene	
Methyl hexadecanoate		Pentylbenzene*	
2-Pentanone*		Methylbenzofuran	
5-Methyltetrahydrofuranone		Naphthalene*	
Methylcyclopentanone		Methyldibenzofuran	10
Ethylmethylcyclopentanone		Dimethylbenzofuran	
(2-Methylfuryl)ethanone		Dibenzofuran	
Indanone*		Dimethylindane	
(3-Ethylnaphthalene)-2-propanone		Methylnaphthalene	6
Furfural*		Methyltetrahydronaphthalene	
Methyltetrahydrofurfural		Ethyltetrahydronaphthalene	
Ethers		Dimethylnaphthalene	
Methyltetrahydrofuran		Propylnapthalene	
Propyldioxolane		Propylallylnaphthalene	
Methoxytoluene		Isopropylnaphthalene	
2-Propylfuran		Isopropylbiphenyl	9
Ethoxybenzene		Dimethylbiphenyl	
Dimethoxybenzene		Diethylbiphenyl	
Ethylmethoxybenzene		Fluorene	
Dimethoxytoluene	4	Methylfluorene	
Methylethyldihydropyran		Anthracene*	
Ethylbutyldihydropyran	5	Pyrene*	
Dimethoxydimethylbenzene		Methylpyrene	
Dimethoxystyrene		Acephenanthrylene	
Diethoxybenzene		Butylvinylnaphthalene	
Dimethoxyethylbenzene		Methylphenyl-2,3-dihydrobenzofuran	12
Dimethoxyethoxybenzene	7	Benzofluorene	
Trimethoxybenzene		Xanthene	
Di-*tert.*-butylmethoxybenzene	8	Methylisopropylnaphthalene	11
Trimethoxybenzene		*Hydrocarbons*	
Trimethoxyallylbenzene		Ethylcyclopentene	
Dimethoxy methyldihydrobenzofuran		Propylcyclopentene	
(Methoxybenzofuryl)styrene		Isopropylcyclopentene	
Aromatics		Isopropylcyclopentane	
Isopropylbenzene*		2-Butylcyclopentane	
Indene*		3-Methyl-4-octene	
Propyltoluene		Dimethylethylcyclohexadiene	
Butylbenzene*		Tetramethylcyclohexadiene	
Isopropyltoluene		Dimethylundecane	
tert.-Butylbenzene*		Tridecane*	
		Tetradecane*	
		Bicyclohexyl	
		Dimethylbicyclohexyl	
		Pentadecane*	
		Hexadecane*	
		Dipropyldodecane	
		Methylheptadecane	

[a] Asterisks indicate compounds identified by comparison with standards.
[b] Peak numbers are those of compounds labelled in Fig. 1.

The organic matrices obtained for the subfractions from size-exclusion chromatography of neutral and acidic fractions are so simple that the simplified procedure described above allowed us to attempt the identification of their likely structures. The structures identified in neutral fractions are reported in Table IV (classification by formulae). A similar classification of weak, medium, strong and very strong acidic fractions is reported in Tables V and VI.

TABLE V

CONSTITUENTS OF THE WEAK ACID FRACTION OF A HORNBEAM PYROLYSIS OIL OBTAINED BY SLOW PYROLYSIS

Compound[a]	Peak No.[b]	Compound[a]	Peak No.[b]
Phenols		*Lactone*	
Phenol*	13	Butyrolactone	
Methylphenol	14	*Esters, aldehydes and ketones*	
Dimethylphenol	16	Acetylfuran	
Ethylphenol		Acetoxymethylbenzofuran	
Methylethylphenol		Acetoxyethylbenzofuran	
Dimethoxyphenol		Methylfurfural	17
Propylphenol		Trimethylbenzaldehyde	
Isopropylphenol	20	Dimethylcyclohexanone	
Trimethylphenol	20	Methylheptanone	
Dimethylmethoxyphenol		*Ethers*	
Methylpropylphenol		Methoxypentane	
Diethylphenol		Dimethyl methoxybenzofuran	
Butenylphenol		Ethylmethoxybenzofuran	
Propenylmethoxyphenol		*Aromatic*	
Propenyldimethoxyphenol		Toluene*	
Trimethoxyphenol			
Propylmethoxyphenol			
Methyl methoxyhydroxybenzoate			
Dimethoxyhydroxybenzaldehyde			
Acetyldimethoxyphenol			
Propionyldimethoxymethylphenol			
Butyryldimethoxyphenol			

[a] Asterisks indicate compounds identified by comparison with standards.
[b] Peak numbers are those of compounds labelled in Fig. 1.

Some comments can be made on the basis of the results in Tables IV–VI. Micro-distillations of each of the fractions (neutral, acidic or basic) were performed at the temperature of the GC analysis. The proportion of compounds characterized under these conditions is estimated to be from 50 to 70%. The improved API 60 method appears to be an efficient technique for acid–base separation. The polarity of the components identified in each of the fractions increases when the acid–base characteristic become greater. Pyrolysis oils obtained after slow pyrolysis of wood contain mainly saturated hydrocarbons and aromatics (neutral fraction), ethers (peculiarly abundant in the neutral fraction), esters (in neutral and weak acid fractions), aldehydes and ketones (in the neutral fraction and weak, medium and strong acid fractions), phenols [containing monophenols (essentially in the weak and medium acid fractions) and naphthols and diphenols, only in the most polar fractions (medium,

TABLE VI

CONSTITUENTS OF THE MEDIUM, THE STRONG AND THE VERY STRONG ACID FRACTIONS OF A HORNBEAM PYROLYSIS OIL OBTAINED BY SLOW PYROLYSIS

Medium acids		Strong and very strong acids	
Compound	Peak No.[a]	Compound	Peak No.[a]
Organic acid		*Organic acids*	
Ethylbenzoic acid		2-Methylbutyric acid	
Naphthols and phenols (di- and mono-)		Methoxyhydroxybenzoic acid	
Ethylnaphthol		*Naphthols and phenols (di- and mono-)*	
Trimethylnaphthol		Dimethylnaphthol	
Ethylhydroxyphenol	19	Diethylnaphthol	
Dimethylhydroxyphenol		Methoxyacetylnaphthol	
Dihydroxyindane		Dihydroxybenzene	18
Methylhydroxy-1,2-dihydronaphthol		Methyldihydroxybenzene	
Methoxyphenol		Acetyldihydroxybenzene	
Allylethoxyphenol		Methylmethoxydihydroxybenzene	
Trimethylmethoxyphenol	22	Methylpropyldihydroxybenzene	
Allylethylphenol		Isopropyldihydroxybenzene	
Propylmethoxyphenol	21	Propyldihydroxybenzene	
Vinylmethoxyphenol		Isobutyldihydroxybenzene	
Allyformylphenol		Methylethyldihydroxybenzene	
Methyl methylhydroxybenzoate	23	Methoxypropyldihydroxybenzene	
Trimethylhydroxyindane		Methylpropionyldihydroxybenzene	
Methylhydroxyindane		Methyl dimethyldihydroxybenzoate	
Hexenylphenol		Methoxydihydroxybenzene	
Ethyldimethylbenzophenone		Methoxyphenol	15
Methyl dimethoxyhydroxybenzoate		Methyl hydroxybenzoate	
Aldehydes and ketones		Hydroxybiphenyl	
Dimethylformylbenzaldehyde		Dimethoxyacetylphenol	
Vinylmethoxybenzaldehyde		Dimethylpropenylphenol	
Trimethylformylbenzaldehyde		Hydroxystilbene	
Trimethoxybenzaldehyde		*Alcohol*	
2-Propylacetophenone		2-Benzofurylethanol	
Dimethylbenzofuranone			
Dimethyldihydrobenzopyranone			
Ethers			
Dimethoxyallylbenzene			
Methyldibenzofuran			
Bifuran			

[a] Peak numbers are those of compounds labelled in Fig. 1.

strong and very strong acid fractions)] and some organic acids in strong and very strong acid fractions. Finally, in addition to these families of which numerous components are isolated from slow pyrolysis oils (see the tables), some families are present but only some of their components are found in pyrolysis oils; these compounds are alcohols and lactones.

In the case of subfractions from basic fractions, the organic matrices are still too complex and our method of identification is inadequate.

CONCLUSIONS

The combination of ion-exchange chromatography and size-exclusion chromatography with coupled capillary GC–MS appears to be suitable for the analysis and the total characterization of pyrolysis oils. The strategy developed allows the accurate characterization of the volatile part of these pyrolysis oils, *i.e.*, about 70% of tars condensed during the pyrolysis. Moreover, this study indicates the efficiency of the functional sorting obtained by means of the improved API 60 technique.

However, the strategy is inadequate for the characterization of nitrogenous bases contained in the condensable tars from wood pyrolysis. Owing to the importance of these nitrogenous bases in relation to the environment, as a consequence of the mutagenic and carcinogenic characteristics of some of them, a new strategy is currently being developed.

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CHROMSYMP. 2100

Identification of flavonoids from *Ginkgo biloba* L., *Anthemis nobilis* L. and *Equisetum arvense* L. by high-performance liquid chromatography with diode-array UV detection

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ABSTRACT

Naturally occurring flavonoids can be separated by reversed-phase high-performance liquid chromatography (HPLC) using 2-propanol and tetrahydrofuran. A development of this approach is described for the HPLC of *Ginkgo biloba*, *Anthemis nobilis* and *Equisetum arvense*. Peaks related to previously reported compounds were identified by co-chromatography with authentic standards and/or by diode-array detection. Tentative assignments of unknown peaks are presented.

INTRODUCTION

Naturally occuring flavonoids (aglycones and glycosides) can be separated and identified using high-performance liquid chromatography (HPLC), particularly reversed-phase HPLC. The separations are rapid and provide high resolution and sensitivity. Because of the analogous structure of most of the investigated compounds, relatively similar solvent mixtures are required to yield satisfactory separations. Methanol–water and acetonitrile–water are the preferred solvent systems.

The addition of acetic or formic acid to these eluents has been described in several papers [1], and frequently sharp separations are achieved only by using gradient elution [2]. Recently, [3] we reported that flavonoids of several medicinal plants can be sharply separated by isocratic elution on C_8 columns with systems containing C_3 alcohols and cyclic ethers. The validity of these systems has been demonstrated for different medicinal plant extracts such as *Betula* [4], *Ononis spinosa* [5] and *Helichrysum italicum* [6]. The present investigation demonstrates that these eluents can separate the flavonols of *Ginkgo biloba*, *Anthemis nobilis* and *Equisetum arvense*. In addition, the use of diode-array detection permits the peaks in the chromatogram to be assigned to previously reported compounds and tentative assignments of unknown peaks to be made.

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EXPERIMENTAL

Materials

2-Propanol and tetrahydrofuran (THF) were of analytical-reagent grade. *Ginkgo biloba* L. leaves, *Anthemis nobilis* L. flowers and *Equisetum arvense* L. leaves were obtained from different commercial sources. A standardized *Ginkgo biloba* extract was purchased from Laboratoires IPSEN. Authentic samples of isoquercitrin, luteolin-7-glucoside, quercitrin, apigenin-7-glucoside, apiin, rutin, kaempferol-3-rutinoside, isorhamnetin-3-rutinoside and sciadopitysin were obtained from Extrasynthese (Genay, France). Astragalin was isolated from *Helichrysum italicum* [6]; bilobetin, isoginkgetin and ginkgetin were prepared from *Ginkgo biloba* leaves [7]. Reference solutions were prepared in methanol (2 mg/ml).

Chromatographic conditions

The liquid chromatograph consisted of a Model U6K universal injector, two Model 510 pumps, a Model 680 automated gradient controller (all from Waters Assoc., Milford, MA, U.S.A.) and a Model HP 1040A photodiode-array detector (Hewlett-Packard, Waldbronn, Germany). The column was C_8 Aquapore RP 300 (250 mm × 4 mm I.D.) (Brownlee Labs., Santa Clara, CA, U.S.A.) with 7-μm spherical particles.

The eluents were as follows: for *Ginkgo biloba*, eluent A water–2-propanol (95:5), eluent B 2-propanol–THF–water (40:10:50), linear gradient from 20% to 60% B in 40 min, flow-rate 2 ml/min; and for *Anthemis nobilis* and *Equisetum arvense*, 2-propanol–water (15:85), flow-rate 2 ml/min.

The acquisition of UV spectra was automatic at the apex, both inflection points and base of all peaks (230–430 nm, 2-nm steps). Peaks of interest were collected by means of a Model 201 fraction collector (Gilson, Biolabo Instruments, Milan, Italy). Hydrolysis of the isolated glycosides were carried out according to the literature [8]. Glucose and rhamnose were detected by gas chromatography [9] as acetyl derivatives.

Sample preparation

Dried *Ginkgo biloba* leaves (600 mg) were extracted for 15 min under reflux with 60% aqueous acetone (15 ml) and the mixture was diluted to 25 ml with the same solvent and filtered through a fritted glass funnel. Prior to injection (5–10 μl), the samples were filtered through a 0.45-μm membrane filter.

Dried *Anthemis nobilis* flowers and *Equisetum arvense* leaves (1 g) were extracted with 15 ml of 60% methanol for 15 min at about 60°C, the clear filtrate was evaporated to dryness under vacuum and the residue was dissolved in 2 ml of methanol. A 1-ml volume of this solution was diluted with 2 ml of water and percolated through a Sep-Pak C_{18} cartridge (preactivated by passing 5 ml of methanol followed by 5 ml of water). After washing with 3 ml of water and 3 ml of 25% methanol, the flavonoid fraction was eluted with 6 ml of 70% methanol. The solvent was evaporated to dryness and the residue was dissolved in 1 ml of methanol. Aliquots of 10–20 μl were injected into the HPLC system.

Purity assay of the chromatographic peaks

The UV spectra of each peak, after subtraction of the corresponding UV base

spectrum, were computer normalized and the plots were superimposed. Peaks were considered to be homogeneous when there was exact correspondence among the corresponding spectra (match factor >990).

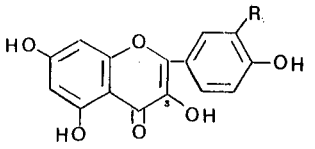
RESULTS AND DISCUSSION

Ginkgo biloba

The constituents of *Ginkgo biloba* leaves have been recently examined [10–12] and many flavonol glycosides have been identified (Table I).

TABLE I

FLAVONOL GLYCOSIDES OF *GINKGO BILOBA* LEAVES



R = OH Quercetin
R = H Kaempferol
R = OCH_3 Isorhamnetin

Type	Compound	No.
Quercetin-3-O-glycosides	Rutin (quercetin-3-O-rutinoside)	I
	Isoquercitrin (quercetin-3-O-glucoside)	II
	Quercitrin (quercetin-3-O-rhamnoside)	III
	Quercetin-3-O-[6‴-*p*-coumaroylglucosyl-(1→2)rhamnoside]	IV
	Quercetin-3-O-rhamnosyl-(1→2)-rhamnosyl-(1→6)-glucoside	V
Kaempferol-3-O-glycosides	Kaempferol-3-O-rutinoside	VI
	Astragalin (kaempferol-3-O-glucoside)	VII
	Kaempferol-3-O-[6‴-*p*-coumaroylglucosyl-(1→2)-rhamnoside]	VIII
	Kaempferol-glycoside	IX
	Kaempferol-3-O-rhamnosyl-(1→2)-rhamnosyl-(1→6)-glucoside	X
Isorhamnetin-3-O-glycosides	Isorhamnetin-3-O-rutinoside	XI

The HPLC of a standardized extract of *Ginkgo biloba* leaves is shown in Fig. 1. Eleven major flavonoids were present (peaks I–XI). Six peaks were identified by comparison with authentic specimens (rutin, I; isoquercitrin, II; quercitrin, III; kaempferol-3-rutinoside, VI; astragalin, VII; and isorhamnetin-3-rutinoside, XI). Together with the retention time data, these peaks were also identified by comparing their UV spectra with those of the corresponding standards. The new spectra of peaks V and X presented a maximum slope in the short-wavelength region [band II, benzoyl typical of quercetin and kaempferol glycosides, respectively (Fig. 2)]. On acid hydrolysis of the isolated compounds, rhamnose and glucose in the ratio 2:1 were detected. These data combined with the short retention times (high polarity) indicated that peaks V and X were quercetin-3-O[rhamnosyl-(1→2)-rhamnosyl-(→6)-glucoside] and kaempferol-3-O-[rhamnosyl-(1→2)-rhamnosyl-(1→6)-glucoside], respectively. They have been previously reported in *Ginkgo biloba* [11,12].

Peak IX gave a spectrum similar to that of kaempferol-3-rutinoside (VI) (Fig. 3). Its resolution time (16 min) compared with that of kaempferol-3-rutinoside (12.3

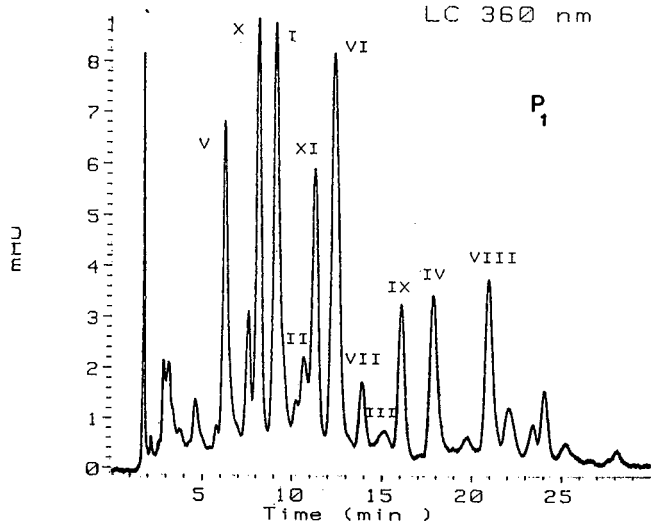


Fig. 1. Typical chromatogram of a standardized extract of *Ginkgo biloba* leaves. Column, 7-μm C_8 Aquapore RP-300; Eluent, (A) water–2-propanol (95:5), (B) 2-propanol–THF–water (40:10:50), linear gradient from 20% to 60% B in 40 min; flow-rate, 1 ml/min. For peaks, see Table I.

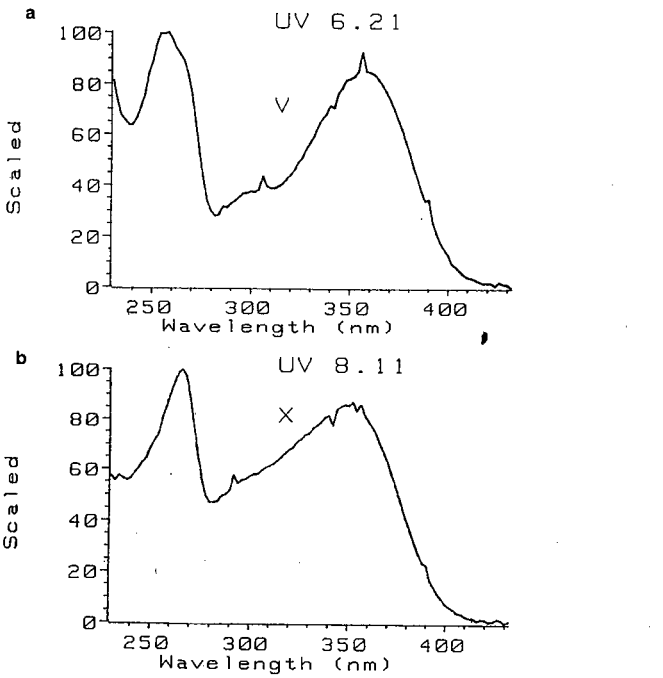


Fig. 2. UV spectra of (a) quercetin-3-O-[rhamnosyl-(1→2)-rhamnosyl-(1→6)-glucoside] (V) and (b) kaempferol-3-O-[rhamnosyl-(1→2)-rhamnosyl-(1→6)-glucoside] (X).

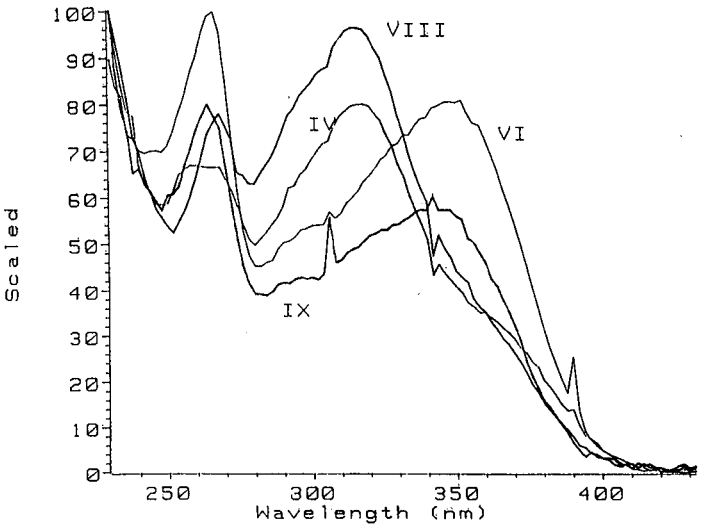


Fig. 3. UV spectra of kaempferol-3-O-rutinoside (VI), peak IX, quercetin-3-O-[6‴-*p*-coumaroylglucosyl-(1→4)-rhamnoside] (IV) and kaempferol-3-O-[6‴-*p*-coumaroylglucosyl-(1→4)-rhamnoside] (VIII).

min) demonstrated that it was less polar. As acid hydrolysis followed by gas chromatographic determination of the liberated sugars yielded glucose and rhamnose, peak IX might reasonably be assumed to be a glucosyl rhamnoside isomer. However, more work is needed for a complete structure elucidation. New peaks IV and VIII showed a band II absorption typical of quercetin and kaempferol glycosides, respectively (Fig. 3). The hypsochromic shift of band I (cinnamoyl ring, λ_{max} = 320 nm, instead of 350–360 nm) indicated the presence of the *p*-coumaroyl group in the side-chain. This finding was confirmed by HPLC determination of *p*-coumaric acid from the isolated peaks after alkaline hydrolysis. Hence, peaks IV and VIII were assigned to the previously identified [10] quercetin-3-O-[6‴-*p*-coumaroylglucosyl-(1→4)-rhamnoside] and kaempferol-3-O-[6‴-*p*-coumaroylglucosyl-(1→4)-rhamnoside], respectively.

A typical chromatogram obtained from *Ginkgo biloba* leaves is shown in Fig. 4. The chromatogram was divided into two parts on the basis of retention times: the first part (7–25 min, similar to that of Fig. 1 and indicated as P_1) consisted of the heterosides I–XI; the second part (P_2) contained the biflavones bilobetin, ginkgetin, isoginkgetin and sciadopitysin. These last were almost absent in the standardized extract.

Anthemis nobilis and Equisetum arvense

Apigenin-7-glucoside, luteolin-7-glucoside and apiin (apigenin-7-apiogluco-side) are the characteristic components of *Anthemis nobilis* flowers [13] and isoquercitrin and quercetin-3-sophoroside are the main constituents of european *Equisetum arvense* [14] (Table II). Well resolved peaks were obtained from extracts of these plants by isocratic elution with 2-propanol–water (15:85). The identity of the peaks from *Anthemis nobilis* L. (Fig. 5) was established by co-chromatography using standard apiin, apigenin-7-glucoside and luteolin-7-glucoside.

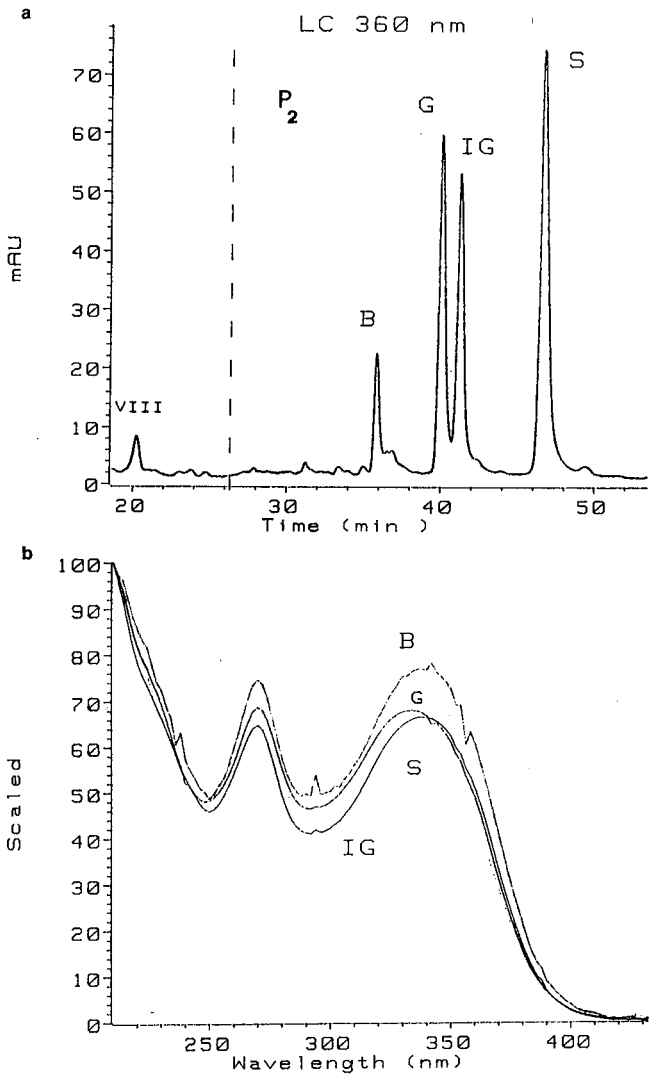
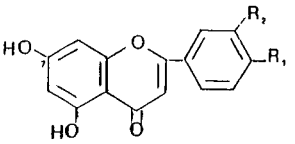


Fig. 4. Typical chromatogram from *Ginkgo biloba* leaves. (a) P_2 (b) UV spectra of bilobetin (B), ginkgetin (G), isoginkgetin (IG) and sciadopitysin (S). See Fig. 1 for P_1 and chromatographic conditions. Linear gradient from 20% to 80% in 60 min.

For *Equisetum arvense* L. (Fig. 6), only the main peak (8.65 min) and the small peak at 14.6 min were identified by co-chromatography with isoquercitrin and astragalin standards, respectively. The other peak (4.71 min) (whose UV spectrum suggested the presence of quercetin) was collected and hydrolysed to yield quercetin and glucose. Therefore, owing to its relatively high polarity this peak was designated as quercetin-3-sophoroside, reported previously [14]. Concerning the peaks at 5.44 and 7.46 min, the first had the spectrum of a cinnamic acid derivative, whereas the second

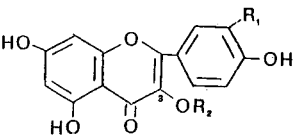
TABLE II

FLAVONES AND FLAVONOL GLYCOSIDES OF *ANTHEMIS NOBILIS* AND *EQUISETUM ARVENSE*



$R_1 = R_2 = OH$ Luteolin
$R_1 = OH$; $R_2 = H$ Apigenin

Species	Compound	No.
Anthemis nobilis	Apigenin-7-O-glucoside	IA
	Apigenin-7-O-apioglucoside	IIA
	Luteolin-7-O-glucoside	IIIA



$R_1 = OH$ Quercetin
$R_1 = H$ Kaempferol

Species	Compound	No.
Equisetum arvense	Isoquercitrin	IE
	Quercetin-3-O-sophoroside	IIE
	Astragalin	IIIE

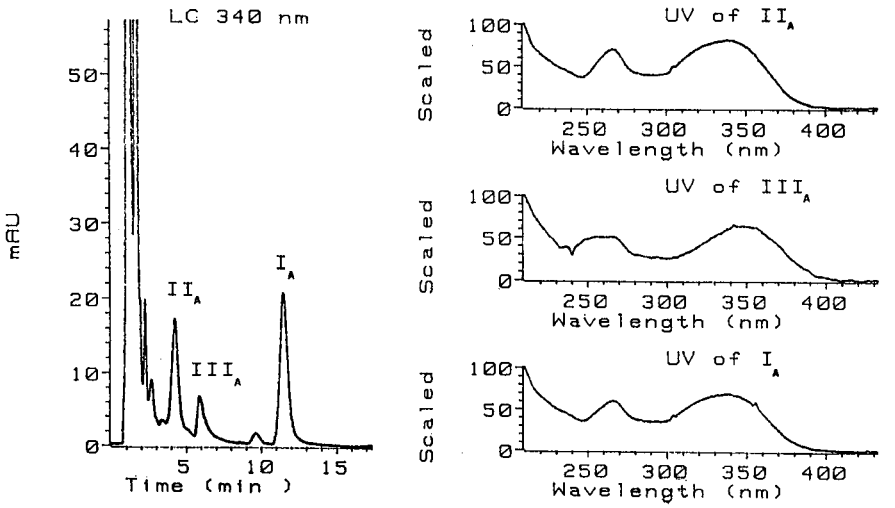


Fig. 5. Typical chromatogram from *Anthemis nobilis* flowers. Column, 7-μm C_8 Aquapore RP-300; eluent, 2-propanol–water (15:85); flow-rate, 2 ml/min. For peaks, see Table II.

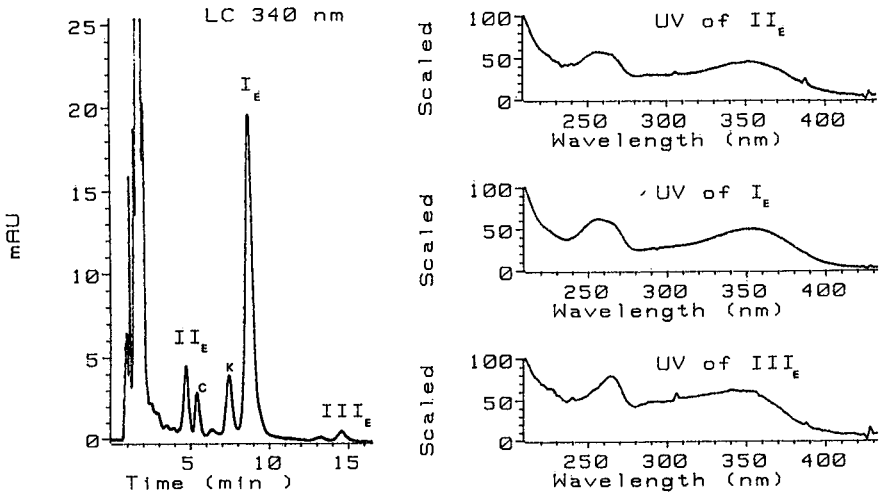


Fig. 6. Typical chromatogram from *Equisetum arvense* leaves. Column, 7-μm C_8 Aquapore RP-300; eluent, 2-propanol–water (15:85); flow-rate, 2 ml/min. For peaks, see Table II.

showed a spectrum typical of kaempferol glycosides (Fig. 7). Further investigation on these peaks was not carried out, as they eluted too closely and in very small amounts.

In conclusion, the combined use of a C_8 column and 2-propanol–tetrahydrofuran–water or 2-propanol–water systems was confirmed to be of great value for the sharp resolution of flavonoids from medicinal plants. Moreover, spectral data obtained with diode-array detection together with the results from the hydrolytic procedures and the retention times allowed the peak to be identified and their purities to be checked.

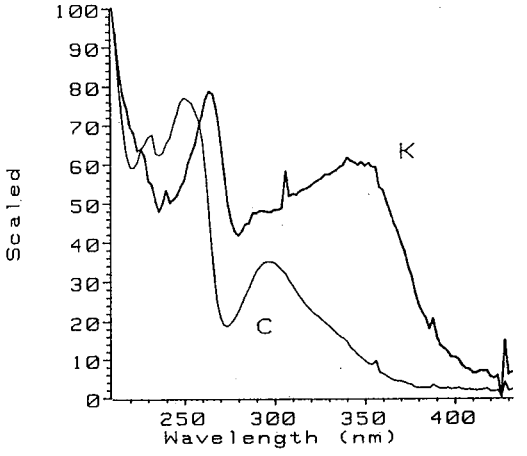


Fig. 7. UV spectra of peaks K and C in Fig. 6.

ACKNOWLEDGEMENTS

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CHROMSYMP. 2209

Measurement of enzyme activities of cytochrome P-450 isoenzymes by high-performance liquid chromatographic analysis of products

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ABSTRACT

A method is described for the qualitative and quantitative determination of the isoenzymes of cytochrome P-450 from rat liver microsomes. Microsomes are incubated with the endogenous steroid 17β-testosterone, which results in the formation of a number of stereo-specific hydroxylation products of testosterone. The hydroxylated products were identified using standards or by comparison with data from the literature. The products can be analysed by reversed-phase gradient high-performance liquid chromatography. The assay has been optimised for pH, linearity and time of incubation. An evaluation of the assay was performed for different kinds of microsomes, microsomal dilution and specificity for particular cytochrome P-450 isoenzymes.

INTRODUCTION

The cytochrome P-450 system consists of a number of isoenzymes which can be induced as a function of the presence of xenobiotic compounds. Each isoenzyme has its own specific function in the metabolism of exogenous and also endogenous compounds [1]. One of the enzymatic reactions which can be performed by cytochrome P-450 are hydroxylations of compounds to increase the polarity or to create possible sites for conjugation [2]. In addition to this detoxifying mechanism, unwanted processes may also occur. Xenobiotic compounds can be activated to reactive metabolites which can be covalently attached to proteins and nucleic acids. In biochemical toxicology the knowledge of the isoenzyme pattern of cytochrome P-450 is an important parameter in the study of organ toxicity. Methods of analysis such as chemiluminescence detection [3] or electrophoretic separation followed by immunochemical detection [4,5] can give information about the isoenzyme pattern. These techniques, however, do not necessarily reflect the real enzyme activity, because only the amount of protein is determined, which is not enzymatically active. Therefore methods are required which can give information about the real enzymatic activity of the individual isoenzymes. Well known specific enzymatic determinations are the

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fluorimetric assays using alkylcoumarins or alkylresorufins as substrates [6]. Several studies have been reported which use steroids as substrates, such as testosterone [7], androstenedione [8], progesterone [9], cholesterol [10] and pregnenolone [11]. In this laboratory a study has been initiated to develop a method in which all known cytochrome P-450 isoenzymes of the rat [12] can be quantitated, based on their enzymatic activity. This paper describes the first part of this study, which is the metabolic activity of cytochrome P-450 isoenzymes towards 17β-testosterone as the substrate.

EXPERIMENTAL

Chemicals

Testosterone and androstenedione were obtained from Sigma, Brunschwig Chemie (Amsterdam, The Netherlands). 6β-, 7α-, 11α-, 11β-, 16α-, 16β- and 19-hydroxytestosterone (OH-T) were from Steraloids, Brunschwig Chemie (Amsterdam, The Netherlands). All other chemicals used were of the highest analytical grade.

High-performance liquid chromatography equipment

The high-performance liquid chromatography (HPLC) equipment consisted of the following components: an automatic injector (Gilson, Model 231), two solvent delivery systems (LKB, Model 2150) controlled by a gradient controller (LKB, Model 2152), a variable-wavelength monitor (LKB, Model 2141) and a computing integration system (LDC/Milton Roy, Model CI-10B). The column used was a Chromsep C_{18} (200 × 3 mm I.D.) with 5 μm particle size (Chrompack, Middelburg, The Netherlands) preceded by a 10 mm × 3 mm I.D. C_{18} guard column (Chrompack).

Preparation of microsomes

Phenobarbital- and β-naphthoflavone-induced male rats. Livers were perfused with ice-cold saline, isolated, weighed and homogenised in ice-cold Tris–potassium chloride using a Potter–Elvehjem glass–PTFE homogeniser. The cell debris, nuclei and mitochondria were removed by centrifugation for 15 min at 16 000 *g* in a Sorvall centrifuge. The supernatant was centrifuged for 60 min at 105 000 *g* in a Centrikon ultracentrifuge. The pellet obtained at this centrifugation step was resuspended in Tris–potassium chloride buffer and again centrifuged for 60 min at 105 000 *g*. The microsomal pellet thus obtained was resuspended in Tris–sucrose buffer containing 25% glycerol, then quickly frozen in liquid nitrogen and stored at −70°C. The cytochrome P-450 content was determined spectrophotometrically according to the method of Omura and Sato [13]. The microsomes contained 1637 and 1290 pmol of cytochrome P-450 per mg of protein for the phenobarbital- and β-naphthoflavone-treated rats, respectively. Protein concentrations were determined according to the method described by Lowry *et al.* [14].

Control and the dexamethasone-induced rats. Livers were perfused with ice-cold saline, isolated, weighed and homogenised in ice-cold phosphate buffer with 1.15% potassium chloride using a Potter–Elvehjem glass–PTFE homogeniser. The cell debris, nuclei and mitochondria were removed by centrifugation for 10 min at 15 000 *g* in a Sorvall centrifuge. The supernatant was centrifuged for 30 min at 150 000 *g* in a Centrikon ultracentrifuge. The microsomal pellet obtained at this step was

resuspended in Tris–sucrose buffer containing 25% glycerol, then quickly frozen in liquid nitrogen and stored at −70°C. The cytochrome P-450 content was determined spectrophotometrically according to the method of Omura and Sato [13]. The microsomes of control rats contained 732 pmol of cytochrome P-450 per mg of protein (male rats), 655 pmol of cytochrome P-450 per mg of protein (female rats) and 1448 pmol of cytochrome P-450 per mg of protein for the dexamethasone-induced male rats. Protein concentrations were determined according to the method described by Lowry *et al.* [14].

In vitro assay

The procedure followed was that of Sonderfan *et al.* [15] with some modifications. The incubation mixture consisted of the following components: potassium phosphate buffer (50 m*M*, pH 7.4), which contained 1 m*M* EDTA, 3 m*M* magnesium chloride, 1 m*M* nicotinamide-adenine dinucleotide phosphate (NADP), 5 m*M* glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, the steroid as substrate (250 μ*M*, final concentration) and 40 μl of microsomes in a total volume of 1 ml. The reaction was started by the addition of the microsomes. After an incubation period of 15 min at 37°C the reaction was stopped by placing the mixture in ice-cold water followed by the addition of 6 ml of dichloromethane. At this point 11β-OH-T was added as an internal standard. After mixing and centrifugation the organic layer containing the metabolites was evaporated under a stream of nitrogen at room temperature. The residue was dissolved in 200 μl of methanol–water (50:50, v/v) for analysis by HPLC.

High-performance liquid chromatographic analysis

The elution conditions were as follows: isocratic elution for 10 min with 11% mobile phase B, then from 10 to 40 min a linear gradient from 11 to 28% B. The flow-rate was maintained at 0.80 ml/min and the column temperature was 50°C. The effluent was monitored at 240 nm. Mobile phase A consisted of methanol–water (30:70, v/v); mobile phase B consisted of methanol–acetonitrile (90:10, v/v). The mobile phases were adjusted to pH 4.5 by the addition of acetic acid.

The quantitation of the testosterone metabolites was performed using the peak heights measured at 240 nm and was corrected for the internal standard 11β-OH-T. The metabolites for which a standard was available were further calculated to concentration units using a standard curve. For the metabolites for which no standard was available, an average absorption efficient of 0.470 (for a solution of 1 μg/ml) was used.

RESULTS

In the first experiment, several endogenous steroids were tested for their ability to serve as substrates for monitoring the isoenzyme pattern of cytochrome P-450. The steroids were incubated with microsomes from control rats, and after extraction, the product formation was monitored with reversed-phase HPLC. The parameters monitored were the extent of substrate consumption of the particular steroid used, the number of metabolites observed in the chromatogram and the relative amounts of the observed metabolites. The following steroids were tested: 17β-testosterone,

3,17-androstenedione, progesterone, 17β-estradiol and cortisol. For 17β-estradiol and cortisol only a few small peaks were observed in the chromatogram. For testosterone, progesterone and androstenedione a number of metabolites were observed, some in relatively high amounts. A total of 28% of the testosterone was metabolised, resulting in four peaks in relatively high amounts (2.7–7.2% relative to the starting amount of testosterone). In addition, eight smaller peaks were observed in the order of 0.25 and 0.6%. Only 7% of the progesterone was degraded, which resulted in four large peaks, ranging from 0.2 to 2.9%, and three smaller peaks. A total of 23% of the androstenedione was metabolised, which resulted in two large peaks of 4.8 and 7.3%, and six smaller peaks in the order of 0.15–0.4% of the starting amount of androstenedione. Based on this information from the chromatograms, 17β-testosterone was selected for further studies.

The assay with 17β-testosterone was developed into a routine assay using microsomes from male rats treated with dexamethasone; these microsomal preparations produced the largest number of peaks in the chromatogram. It is shown in Fig. 1 that, in addition to the internal standard 11β-OH-T, eleven peaks can be observed which are not found in the blank experiment. Three or four very small peaks can also be observed. By comparing these with various hydroxylated testosterone standards which were obtained commercially, seven peaks could be identified: 6β-OH-T at 9.45 min, 19-OH-T at 10.35 min, 7α-OH-T at 11.28 min, 16α-OH-T at 15.38 min, 16β-OH-T at 17.63 min, 11α-OH-T at 19.18 min and androstenedione at 33.73 min. The peaks with retention times of 23.26 and 25.03 min could be assigned to 2α-OH-T and 2β-OH-T, respectively, by comparison with literature data [8,10] in combination with

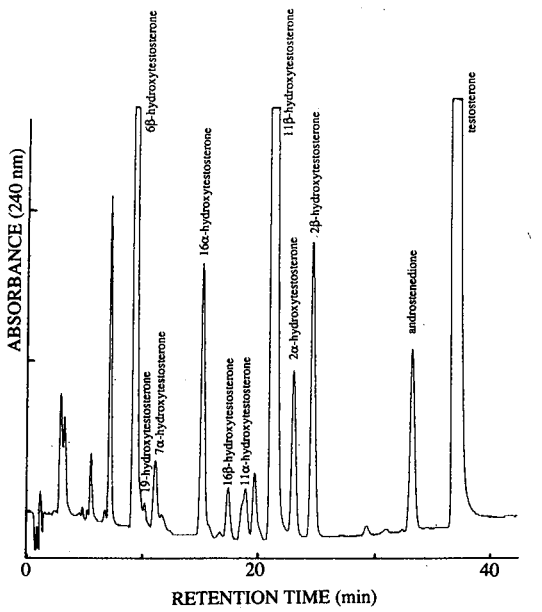

Fig. 1. HPLC pattern of the incubation mixture of 17β-testosterone with microsomes of a rat treated with dexamethasone. The identified hydroxylated testosterone metabolites are indicated in the chromatogram.

the results obtained with microsomes from control rats. The 2α-OH-T peak is large in control microsomes and decreases in intensity on induction by dexamethasone, phenobarbital and β-naphthoflavone. The 2β-OH-T peak shows a low intensity in the control microsomes, whereas an increase is observed on induction by dexamethasone and phenobarbital. The relative retention times are in agreement with reported data. The remaining major peaks that are still to be identified have retention times of 5.71, 7.31 and 19.98 min, there are small unidentified peaks at 11.86 and 29.76 min.

The quantitation of the metabolites was performed by comparison of the HPLC peak heights with those of available standards. Hydroxylated testosterone metabolites for which no standard was available (2α- and 2β-OH-T) were quantitated using an average absorption coefficient of 0.470, which was calculated from seven hydroxylated testosterone standards. The absorption coefficients and maximum wavelengths are summarised in Table I. For all standards the working range was checked and showed a perfectly linear behaviour between 50 and 800 ng of the steroid injected, with correlation coefficients in the range 0.995–0.999. The limit of detection for the hydroxylated steroids was about 3 ng per injection with a signal-to-noise ratio of 3. This value was extrapolated from a 50-ng amount.

TABLE I

MAXIMUM WAVELENGTH AND ABSORPTION COEFFICIENTS (1 μg/ml SOLUTIONS) OF 17β-TESTOSTERONE AND METABOLITES

Steroid[a]	Absorption coefficient at 240 nm	Maximum wavelength (nm)	Maximum absorption coefficient
T	0.565	247.8	0.647
6β-OH-T	0.499	241.2	0.505
7α-OH-T	0.555	247.0	0.623
11α-OH-T	0.485	247.4	0.542
11β-OH-T	0.350	247.2	0.393
16α-OH-T	0.388	248.6	0.462
16β-OH-T	0.478	247.5	0.540
19-OH-T	0.388	248.6	0.462
AD	0.553	246.1	0.600

[a] T = 17β-testosterone; AD = 3,17-androstenedione.

The pH dependence of the hydroxylation of testosterone was investigated for some metabolites using microsomes from a control male rat. Table II gives the results for pH values 6.9, 7.4, 7.9 and 8.4. It appears that the various stereo-specific hydroxylation reactions show different pH behaviours. For all further experiments a pH of 7.4 was chosen as a compromise.

The kinetics of the hydroxylation reaction was investigated by monitoring the relative peak heights as a function of time. The results of this experiment are shown in Fig. 2 for the hydroxylation of testosterone by microsomes from male rats which have been treated with dexamethasone. All the curves showed a linearity from 0 to 15 min. An incubation time of 15 min was therefore chosen for all further experiments.

TABLE II

pH DEPENDENCE OF THE FORMATION OF METABOLITES OF 17β-TESTOSTERONE

Determined with microsomes from a control male rat. Data are expressed in arbitrary units as peak heights, corrected for the internal standard 17β-OH-T.

Metabolite	Peak height			
	6.9	7.4	7.9	8.4
2α-OH-T	409	469	371	341
2β-OH-T	13.2	26.8	31.6	32.7
6β-OH-T	202	526	681	746
7α-OH-T	109	127	89	77
16α-OH-T	531	672	581	539
16β-OH-T	20.3	31.2	27.4	27.1
AD[a]	585	666	618	597

[a] 3,17-Androstenedione.

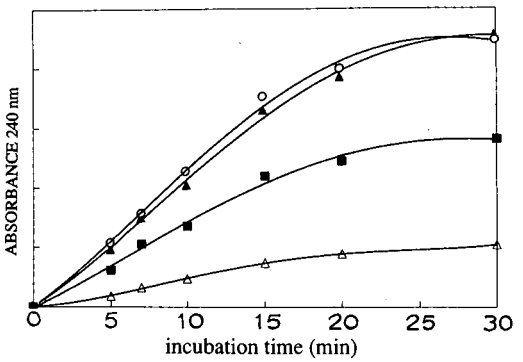

Fig. 2. Kinetic behaviour of the formation of four metabolites of 17β-testosterone as a function of incubation time as determined with microsomes from a male rat treated with dexamethasone. (■) 2α-OH-T; (△) 7α-OH-T; (▲) 6β-OH-T; and (○) 16β-OH-T.

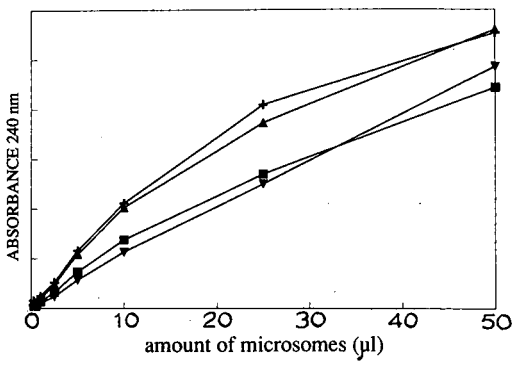

Fig. 3. Linearity of the formation of four metabolites as a function of the volume of rat liver microsomes in the range 0.5–50 μl. (+) Androstenedione; (▲) 16α-OH-T; (■) 2α-OH-T; and (▼) 6β-OH-T.

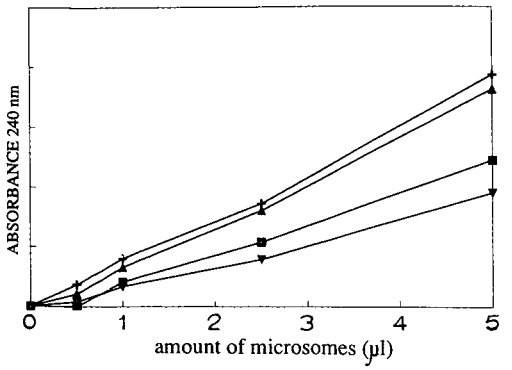

Fig. 4. Linearity and determination of the limit of detection of the formation of four metabolites as a function of the volume of rat liver microsomes in the range 0.5–5 µl. Symbols as in Fig. 3.

To determine the linearity on dilution and the limits of detection with respect to the amount of microsomes, dilution experiments were performed with microsomes from both control male rats and male rats treated with dexamethasone. An approximate linear behaviour was obtained, with 50 µl of microsomes as an upper and 5–0.5 µl of microsomes as a lower detection limit, depending on the peak height (Fig. 3). High-intensity peaks could be observed down to a 200-fold dilution of the microsomal preparations in the buffer, resulting in an absolute amount of 0.5 µl of microsomes (Fig. 4), whereas peaks of low intensity could be detected with 5 µl of microsomes. The criterion used for peak detection was the indication of peaks by the integrator. In practice the limits of detection can be decreased further by changing the integration parameters. An example of such a dilution experiment is shown in Fig. 4 for four HPLC peaks after the incubation of testosterone with microsomes from a control rat.

DISCUSSION

The occurrence of the various cytochrome P-450 isoenzymes can be monitored by product formation in a real enzymatic assay in addition to other analytical detection techniques such as electrophoresis followed by Western blotting with immunochemical detection. Steroids were shown to be good substrates for the cytochrome P-450 system. 17β-Testosterone and progesterone are good substrates which can be hydroxylated at various stereochemically different sites. HPLC proved to be very suitable to detect and quantitate the various metabolites. The results of this study indicate that twelve peaks can be observed in a simple reversed-phase gradient system. By comparison with standards, eight of these peaks could be assigned to several hydroxylated isomers of testosterone and to androstenedione. Two more peaks could be identified based on comparison with data from the literature, combined with experiments with microsomes from control rats. A few peaks still remain to be identified.

In addition to a number of cytochrome P-450 isoenzymes, the rat liver microsomes also contain other enzymes which can act on endogenous steroids, such as

5α-reductase. This enzyme renders the 3-keto-4-ene moiety into a saturated A-ring structure. The resulting 3-keto- or 3-hydroxyandrostanes cannot be detected by HPLC because of the absence of a sensitive ultraviolet-absorbing group. A predominant activity of 5α-reductase is present, especially in microsomes from female rats [16]. As a result, the assay described here cannot be used for the assessment of the cytochrome P-450 isoenzymes in female rats. Incubation of testosterone with microsomes from female rats results in complete consumption of testosterone and the formation of androstanes, which results in an "empty" chromatogram. Although the activity of 5α-reductase is much lower in male rats, the influence of this enzyme cannot be completely ignored. Possible effects on the quantitative determination of different cytochrome P-450 isoenzymes can be expected. Therefore the addition of 5α-reductase inhibitor is probably a prerequisite for good quantitation of the real enzymatic activity.

To assign the formation of the different hydroxylated testosterone metabolites to the presence of certain specific isoenzyme forms of cytochrome P-450, a large amount of data from the literature has been combined. Isoenzyme P-450a (new nomenclature: IIA1 [12]) is specifically involved in the formation of 7α-OH-T [15,17–21]. Isoenzyme P-450b (IIB1) metabolises testosterone into the metabolites 16α-OH-T, 16β-OH-T and androstenedione. The formation of 16β-OH-T is specific for this isoenzyme. Isoenzyme P-450c (IA1) hydroxylates testosterone on the 6β- and 2β-position [15,17,20]. As this isoenzyme has a very low hydroxylase activity towards testosterone, the contribution to the metabolic pattern will be low. For isoenzyme P-450d (IA2) the same, but lower, activities were found as for isoenzyme c. As a result, isoenzymes c and d, which are important in the assessment of exposure to polychlorinated hydrocarbons, cannot be detected with the testosterone assay. Isoenzyme P-450e (IIB2) shows the same metabolic pattern as isoenzyme b, but has only about 10% of its activity [20]. With isoenzyme P-450g (IIC13) the metabolites 6β-OH-T, 15α-OH-T and 16α-OH-T are formed, but in low amounts [17]. Isoenzyme P-450h (IIC11) catalyses the specific formation of 2α-OH-T and also the less specific metabolite 16α-OH-T [15,17–19]. Isoenzyme P-450p (IIIA1) forms four hydroxylated metabolites of testosterone, 2β-OH-, 6β-OH-, 15β-OH- and 18-OH-T [15,19]. As this isoenzyme is responsible for more than 85% of the 6β-hydroxylase activity, the occurrence of this metabolite can be ascribed to isoenzyme P-450p.

In conclusion, it can be stated that with the testosterone hydroxylase assay the following cytochrome P-450 isoenzymes can be quantitated based on their enzymatic activity: isoenzymes P-450 a (7α-OH-T), b (16β-OH-T), h (2α-OH-T) and p (6β-OH-T). For this very sensitive assay only a limited amount (0.5 μl) of microsomes is required. Further studies, including more statistical evaluations and practical applications, will be performed as soon as inhibitors of 5α-reductase become available.

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CHROMSYMP. 2150

Determination of ethylenediaminetetraacetic acid and its salts in canned mushrooms by reversed-phase ion-pair liquid chromatography

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ABSTRACT

A method for the determination of ethylenediaminetetraacetic acid (EDTA) in the surrounding liquid of canned mushrooms, based on reversed-phase ion-pair high-performance liquid chromatography (HPLC), was developed. Sample pretreatment simply consists in addition of copper(II) chloride and ascorbic acid to the filtered sample and microfiltration prior to injection into the HPLC system. The copper(II)–EDTA complex thus formed is separated with a water–methanol mobile phase containing tetrabutylammonium as the counter ion at pH 4.0 and UV detection is applied at 300 nm. Iron(III) concentrations up to 33 mg/l do not interfere. The recovery is 105.6 ± 3.0% at a level of 18 mg/l EDTA and 101.5 ± 0.8% at a level of 178 mg/l EDTA. The method is linear in the range 7–178 mg/l EDTA. The repeatability of the procedure is 5 mg/l EDTA (n = 20). The limit of determination is 10 mg/l EDTA. The day-to-day (n = 10) relative standard deviation is 2.3%.

INTRODUCTION

Enzymatic browning is a negative quality aspect of edible mushrooms. It is an indirect result of the action of the copper-containing enzyme polyphenol oxidase (PPO), which can react with some hormones to form melanin. EDTA is a complexing agent which may inactivate the action of PPO by sequestering copper [1]. Consequently, enzymatic browning may be inhibited by the addition of EDTA. However, in the Netherlands the addition of EDTA to canned mushrooms is prohibited except for export to the U.S.A.

Various methods have been described for the determination of EDTA in different matrices, including colorimetry [2], titrimetry [3], anion-exchange high-performance liquid chromatography (HPLC) [4] and reversed-phase ion-pair HPLC [5–7]. In the last technique, tetrabutylammonium was used as the counter ion and EDTA was separated as its copper complex [5] or as its iron(III) complex [6,7], which was detected by UV absorption at 254 nm [5,6], 245 or 300 nm [7].

In our hands, the colorimetric method that was described for food products, including canned mushrooms [2], yielded very disappointing results, even if applied to standard solutions, and with the titrimetric method discrimination between EDTA and other chelates is not possible. For this reason, a reversed-phase ion-pair HPLC method was developed, based on the method described by De Kleijn *et al.* [7].

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EXPERIMENTAL

Chemicals and reagents

Ethylenediaminetetraacetic acid, calcium disodium salt dihydrate (Ca-EDTA), was obtained from Sigma. Tetrabutylammonium hydroxide (20% aqueous solution) was purchased from Merck. All other chemicals were of analytical or reagent grade (Merck) and were used without further purification. Water was purified with a Milli-Q water-purification system (Millipore). Copper(II) chloride solution was prepared by dissolving 110 mg of copper(II) chloride dihydrate in 30 ml of glacial acetic acid and diluting with water to a final volume of 100 ml. Iron(III) chloride solution was prepared by dissolving 175 mg of iron(III) chloride hexahydrate in 30 ml of glacial acetic acid and diluting with water to a final volume of 100 ml. A stock standard solution was prepared by dissolving 1 g of Ca-EDTA in 100 ml of water. From this stock solution calibration standards were prepared in water containing 10, 50, 100 and 250 mg/l Ca-EDTA.

Chromatography

The apparatus consisted of the following parts: a Waters Model 6000 A liquid chromatographic pump, a Merck/Hitachi 655A UV–VIS detector, a Rheodyne Model 7125 syringe-loading sample injector, a Kontron Model 480 oven controller and an Olivetti personal computer with Nelson Integration software.

The analytical column was LiChrosorb 5-RP-18 (150 × 4.6 mm I.D.) (Chrompack). The precolumn was Chromguard C_{18} (10 × 3.0 mm I.D.) (Chrompack). The mobile phase was the same as that applied by De Kleijn *et al.* [7] and was prepared by mixing 1 l of 0.03 *M* sodium acetate–acetic acid buffer (pH 4) containing 20 m*M* tetrabutylammonium hydroxide with 100 ml of methanol. Before use the mobile phase was filtered using a Millipore solvent clarification kit equipped with a 0.45-μm HV filter.

The chromatographic conditions used throughout this study were: flow-rate 1.0 ml/min, injection volume 20 μl and column temperature 50°C. Unless stated otherwise, the detection wavelength was 300 nm. Prior to analysis the chromatographic system was conditioned overnight with the mobile phase.

Sample preparation

The surrounding liquid of canned mushrooms was filtered through Schleicher & Schüll paper (595½). Unless stated otherwise, to 22.5 ml of the filtered sample 2.5 ml of the copper(II) chloride solution and 20 mg of L-(+)-ascorbic acid were added. After thorough mixing and microfiltration over a 0.45-μm Acrodisc disposable filter assembly (Gelman Sciences), the filtrate was ready for injection into the HPLC system.

Calibration and quantification

Calibration graphs were obtained by least-squares analysis using calibration standards containing 10, 50, 100 and 250 mg/l Ca-EDTA. Prior to injection into the HPLC system, these standards were pretreated in the same way as the samples except for paper filtration and addition of L-(+)-ascorbic acid.

In daily use, quantification was performed by comparison of the peak area of a sample with that of a calibration standard containing 100 mg/l Ca-EDTA. All

concentrations are expressed in mg/l EDTA by multiplying the concentration of Ca-EDTA by a factor of 0.712.

RESULTS AND DISCUSSION

Complexation with iron(III) or copper(II)

EDTA can be complexed with, among other cations, iron(III) and copper(II) ions [7,8]. In reversed-phase ion-pair chromatography, the retention of the Cu(II)–EDTA complex is larger than that of the Fe(III)–EDTA complex [7]. Under the conditions described under Experimental, the retention time of the former complex was about 8.5 min (see Fig. 1a) while that of the latter was about 3.5 min.

Using the same mobile phase and a similar type of analytical column, De Kleijn *et al.* [7] determined EDTA in mayonnaise as the Fe(III)–EDTA complex after extraction with dichloromethane. Applying the sample preparation described under Experimental, in most of the samples of canned mushrooms the Fe(III)–EDTA peak was not completely separated from one or more matrix peaks. In our hands, it was not possible to improve this separation by changing the composition of the mobile phase, *i.e.*, by decreasing the methanol content or by increasing the tetrabutylammonium concentration.

For the Cu(II)–EDTA peak no interferences from matrix peaks were observed (see Fig. 1b), so copper(II) was selected for complexation.

Wavelength selection

Investigations on wavelength selection were performed through analysis of several samples of canned mushrooms, applying detection at 245, 270 or 300 nm. Although the molar absorptivity of the Cu(II)–EDTA complex is smaller at 300 nm than at 245 or 270 nm, the best results were obtained at 300 nm with regard to selectivity (see Fig. 1b) and consequently 300 nm was selected as the detection wavelength.

Interference from iron(III) ions

The stability constant of the Fe(III)–EDTA complex ($\log k_1 = 25.1$) is higher than that of the Cu(II)–EDTA complex ($\log k_1 = 18.8$) [8]. Consequently, the determination of EDTA suffers interference if iron(III) ions from the surrounding liquid of canned mushrooms and/or from the HPLC system are present. This interference can be overcome by the addition of L-(+)-ascorbic acid to the surrounding liquid of canned mushrooms.

L-(+)-Ascorbic acid reduces iron(III) to iron(II), which forms a weaker complex with EDTA ($\log k_1 = 14.3$) [8] than copper(II).

In order to check the effect of L-(+)-ascorbic acid, the following experiment was performed. After complexation of a standard containing 18 mg/l EDTA with copper(II) according to the normal procedure, iron(III) ions were added at a concentration of 33 mg/l [about five times the maximum concentration in the liquid of canned mushrooms ($n = 20$)], resulting in total disappearance of the Cu(II)–EDTA peak after a waiting time of 20 min (the waiting time is necessary because of the relatively slow reaction rates of the complex formation and dissociation reactions [8]). After addition of L-(+)-ascorbic acid (20 mg to 25 ml) the Cu(II)–EDTA complex was formed again quantitatively.

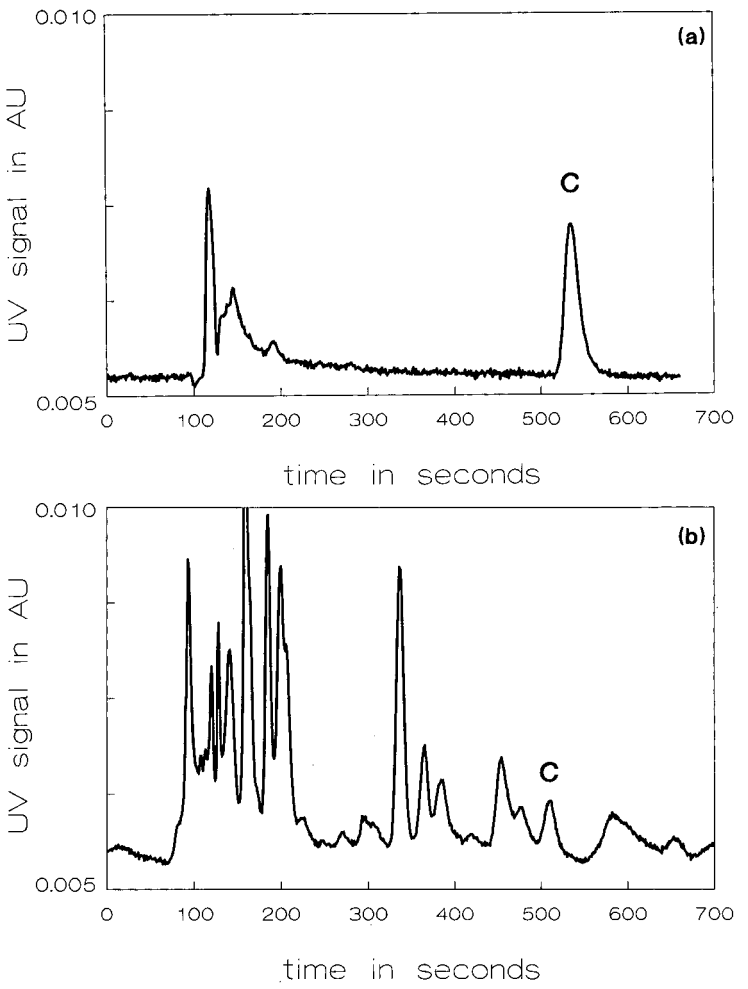

Fig. 1. Complexation of EDTA with copper(II). (a) Standard solution containing 18 mg/l EDTA; (b) sample of surrounding liquid of canned mushrooms containing 8 mg/l EDTA. For sample preparation and chromatographic conditions, see Experimental. C = Cu(II)–EDTA complex.

Confirmation of the presence of EDTA

The difference between the stability constants of the Cu(II)–EDTA and Fe(III)–EDTA complexes can be exploited for confirmation of the presence of EDTA in the surrounding liquid of the canned mushrooms. If, applying the normal sample preparation described under Experimental, a Cu(II)–EDTA peak is detected, this peak will disappear after addition of iron(III) chloride to the filtrate ready for injection into the HPLC system. The volume of the iron(III) chloride solution (see Experimental) to be added has to be adjusted to the presence of L-(+)-ascorbic acid in the filtrate. Assuming complete stability, 35 ml of iron(III) chloride solution would be required for complete oxidation of 20 mg of L-(+)-ascorbic acid [10]. However, owing to the instability of L-(+)-ascorbic acid in aqueous solutions, in our experience the addition

of 5 ml of iron(III) chloride was sufficient for complete dissociation of the Cu(II)–EDTA complex (again a waiting time of 20 min was necessary).

Determination of salts of EDTA

It is assumed that if EDTA is added to canned mushrooms in agro-industrial practice it is applied as the calcium disodium salt. However, if EDTA is added as such or as another salt, it will still be determined with the method described here, provided that the stability constant of the salt is smaller than that of the Cu(II)–EDTA complex. This holds true for most salts, *e.g.*, the Na^+, Li^+ and Ba^{2+} salts [8,9]. For salts with higher stability constants, such as Co(III)–EDTA and Bi(III)–EDTA [8], sequestration of copper in PPO (see Introduction) will not occur and consequently these salts do not prevent enzymatic browning.

Quantitative aspects

The linearity of the method was investigated with calibration standards containing 10, 50, 100 and 250 mg/l Ca-EDTA, corresponding to 7–178 mg/l EDTA. For both peak-area and peak-height measurements linear calibration graphs were obtained over the whole range, with correlation coefficients of 0.9999 and intercepts corresponding to EDTA concentrations < 1 mg/l.

The detection limit, based on a signal-to-noise-ratio of 3, was 1.4 mg/l EDTA. The limit of determination, defined as the mean concentration of EDTA in blank samples ($n = 20$), increased with six times the standard deviation ($\bar{x} + 6\sigma_{n-1}$), was 10 mg/l EDTA. The recovery was $105.6 \pm 3.0\%$ ($n = 10$) and $101.5 \pm 0.8\%$ ($n = 9$) at concentrations of 18 and 178 mg/l EDTA, respectively.

The repeatability, as calculated from the differences between the results of duplicate determinations on 20 samples containing EDTA at concentrations in the range 9–114 mg/l, was 5 mg/l. The day-to-day ($n = 10$) relative standard deviation was 2.3% at a concentration of 79 mg/l EDTA.

Determination of EDTA in canned mushrooms

Applying the method described here, the EDTA concentrations in about 150 samples of canned mushrooms were determined. These samples were produced by almost all Dutch suppliers. If EDTA concentrations exceeded the limit of determination (10 mg/l) the results were verified by means of a titrimetric method [3], which yielded corresponding results. During a 3-month period, about 300 sample solutions were injected onto the same chromatographic column; the retention time of the Cu(II)–EDTA peak gradually decreased from about 9 to 6 min. However, the column efficiency and the resolution between the Cu(II)–EDTA peak and surrounding matrix peaks were not negatively affected.

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High-performance liquid chromatographic determination of acetone in blood and urine in the clinical diagnostic laboratory

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ABSTRACT

A method for the determination of acetone in plasma or urine by high-performance liquid chromatography (HPLC) was developed. Plasma specimens are deproteinized with acetonitrile (1:1, v/v); 2,4-dinitrophenylhydrazine (DNPH) is added to the supernatant or to filtered urine samples, similarly treated with acetonitrile (2:1, v/v) to prevent crystallization of the synthesized phenylhydrazone. An aliquot (20 μl) of the reaction mixture was subjected to HPLC at ambient temperature using a reversed-phase Pecosphere 3×3 C_{18} column with acetonitrile–water (45:55, v/v) as eluent at a flow-rate of 1 ml/min and detection at 365 nm. Hydroxyacetone and acetoacetate phenylhydrazone derivatives do not interfere. The identification of acetone by its retention time was confirmed by comparison with a laboratory-synthesized acetone DNPH derivative. The concentration of acetone, eluted within 3 min, was determined by the peak-height method. The detection limit was 0.034 mmol/l; the relative standard deviations were $< 5\%$ within run (n = 20) and $< 10\%$ between run (n = 20).

INTRODUCTION

In conditions that limit carbohydrate utilization with a resulting increase in fat utilization (diabetes mellitus, starvation, alcoholism), ketone bodies (acetone, acetoacetate and β-hydroxybutyrate) are produced by the liver [1–4]. Acetone, which is formed spontaneously from acetoacetate by irreversible decarboxylation, seems to play a minor role, but some reports over the last 10 years have indicated a renewed interest in its metabolism and there is a need for a method for its measurement, free from interferences [5]. Moreover, acetone has recently become recognized in plasma [6] and especially in urine [7,8] as an indicator of occupational exposure to acetone or isopropanol [9]. The exposure to acetone is evaluated by measurement of the ketone itself in urine in which the ketone pressure is in equilibrium with the pressure in the blood and alveoli [7,8]. In the biological monitoring of exposure to isopropanol, the urinary acetone is present as its catabolite.

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Chemical methods used to test acetone often lack in specificity [10,11]. Enzymatic methods are more specific but more complex and have long assay times and gas chromatographic methods, although widely used, are applied with difficulty as routine tests [12–20]. Here we describe a rapid and simple high-performance liquid chromatographic (HPLC) procedure that can be used for the routine measurement of acetone in biological fluids, such as plasma and urine. The method is based on the derivatization of acetone with 2,4-dinitrophenylhydrazine (DNPH), using very small amounts of sample; the derivative concentration is rapidly obtained without recourse to a solvent extraction step. Reference ranges in plasma and urine for healthy subjects were determined and data obtained for diabetic subjects and exposed workers are reported.

EXPERIMENTAL

Reagents and solutions

All reagents were of analytical-reagent grade. Deionized water was distilled in an all-glass still. The following chemicals were obtained from Sigma (St. Louis, MO, U.S.A.): hydroxyacetone, sodium acetoacetate, DNPH and acetonitrile. Acetone and hydrochloric acid were obtained from Aldrich (Milwaukee, WI, U.S.A.).

DNPH reagent. A 0.25-g amount of DNPH was dissolved in a hydrochloric acid–distilled water (40:60, v/v) mixture by warming in a water-bath at 60°C for 20 min.

Stock solution of acetone DNPH derivative. The phenylhydrazone was synthesized in crystalline form from a mixture of a 10% aqueous acetone solution and DNPH reagent (75:25, v/v). The crystalline product, separated by centrifugation (1800 *g* for 15 min) was washed twice with deionized water and recrystallized from ethanol as described elsewhere [21]. The DNPH derivative was dissolved in acetonitrile and, by injection into the HPLC system, was utilized for retention time control.

Working standard solutions. Calibration graphs were prepared by adding known amounts of acetone (0.107, 0.215, 0.430, 0.860, 1.720 and 3.440 mmol/l) to plasma or urine specimens of healthy unexposed men.

Sample collection and preparation

Blood was collected, with K_2EDTA as anticoagulating agent, from diabetic patients, workers exposed to acetone and healthy unexposed men. As soon as possible after collection, the plasma was separated in a refrigerated (4°C) centrifuge (1000 *g* for 5 min) and either analysed immediately or stored at −20°C to minimize decarboxylation of acetoacetate and loss of acetone and thawed at 4°C just before analysis.

Urine specimens, kept in well closed bottles, were obtained from healthy unexposed men and from workers exposed to acetone. The samples, filtered through 0.2-μm poresize disposable filters (Millepore, Milford, MA, U.S.A.), were stored at 4°C for 48 h or at −20°C for longer periods.

Volumes 200 μl of each plasma sample or standard were added to 1.5-ml centrifuge tubes containing 200 μl of acetonitrile and the tubes were stoppered, vortex mixed (5 s) and centrifuged at 4°C (1000 *g* for 5 min). A 200-μl volume of the supernatant was transferred to another tube and 40 μl of DNPH reagent were added. The mixture was vortex mixed for 5 s and, after 5 min, 20-μl aliquots were injected at room temperature into the HPLC system and the peak heights read at 365 nm.

A volume of 500 μl of each filtered urine sample or standard was added to a tube containing 250 μl of acetonitrile and 200 μl of DNPH reagent. The mixture was vortex mixed for 5 s and, after 5 min, 20-μl aliquots were used for chromatography.

Chromatography

HPLC separation and peak detection of acetone DNPH derivative were carried out on a Pecosphere 3 × 3 C_{18} (3 μm packing) reversed-phase column (3.3 cm × 4.6 mm I.D.) in an HPLC system consisting of a Series 410 LC pump and an LC 90 UV ultraviolet spectrophotometer (both from Perkin-Elmer, Norwalk, CT, U.S.A.), coupled to an integrator developed by S.P.E. Sistemi e Progetti Elettronici (Brescia, Italy). The mobile phase was acetonitrile–water mixture (45:55, v/v) at a flow-rate of 1.0 ml/min.

Statistics

The within-days relative standard deviation (R.S.D.) was based on the analysis of twenty samples prepared from a pooled specimen of plasma or urine, all samples being extracted and analysed during the same day. The between-days R.S.D. was based on the analysis of single samples obtained from the same plasma or urine pool (stored at −20°C and thawed at 4°C just before acetone determination) on 20 separate days. Calibration graphs were prepared for each between-day analysis. Single injections of samples and standards were made. The means and standard deviations were calculated to determine the within-day and between-day R.S.D.s.

RESULTS

Fig. 1 shows chromatograms of (a) a plasma sample and (b) a urine sample from an exposed subject. Hydroxyacetone and acetoacetate DNPH derivatives do not interfere; the first derivative has a retention time of 57 s (Fig. 2) and the peak of the second derivative is not eluted under the chromatographic conditions described.

The identity of the acetone DNPH derivative peak was confirmed by the retention time of a laboratory-synthesized acetone DNPH derivative dissolved in acetonitrile. The calibration graphs (obtained by adding known amounts of acetone) were linear for both plasma and urine up to at least 3.440 mmol/l and were represented by the regression equations $y = 1.97 + 123.8x$ for plasma and $y = 0.366 + 66.3x$ for urine, where y = peak height (mV) and x = acetone concentration (mmol/l). The lower sensitivity in the urine is due to the presence of other ketonic and aldehydic groups which compete with acetone for DNPH. The addition of more DNPH or dilution of the urine did not improve the sensitivity.

The limit of detection in both plasma and urine at a signal-to-noise ratio of 2 was 0.034 mmol/l. The within-run and between-run R.S.D.s were 3.8% and 7.4%, respectively, for plasma and 4.8% and 9.2%, respectively, for urine ($n = 20$), using a pooled plasma and urine specimen containing 0.172 mmol/l of acetone. Instrumental variation accounted for about 2% of the total variation.

The reference acetone values [$x \pm 2\,s$ (s = standard deviation)] for healthy unexposed men were 0.034–0.120 mmol/l with a mean value of 0.075 mmol/l in plasma ($n = 20$) and 0.034–0.095 mmol/l with a mean value of 0.052 mmol/l in urine ($n = 20$). These compare well with the reference values suggested by other workers and obtained by gas chromatographic methods [22,23].

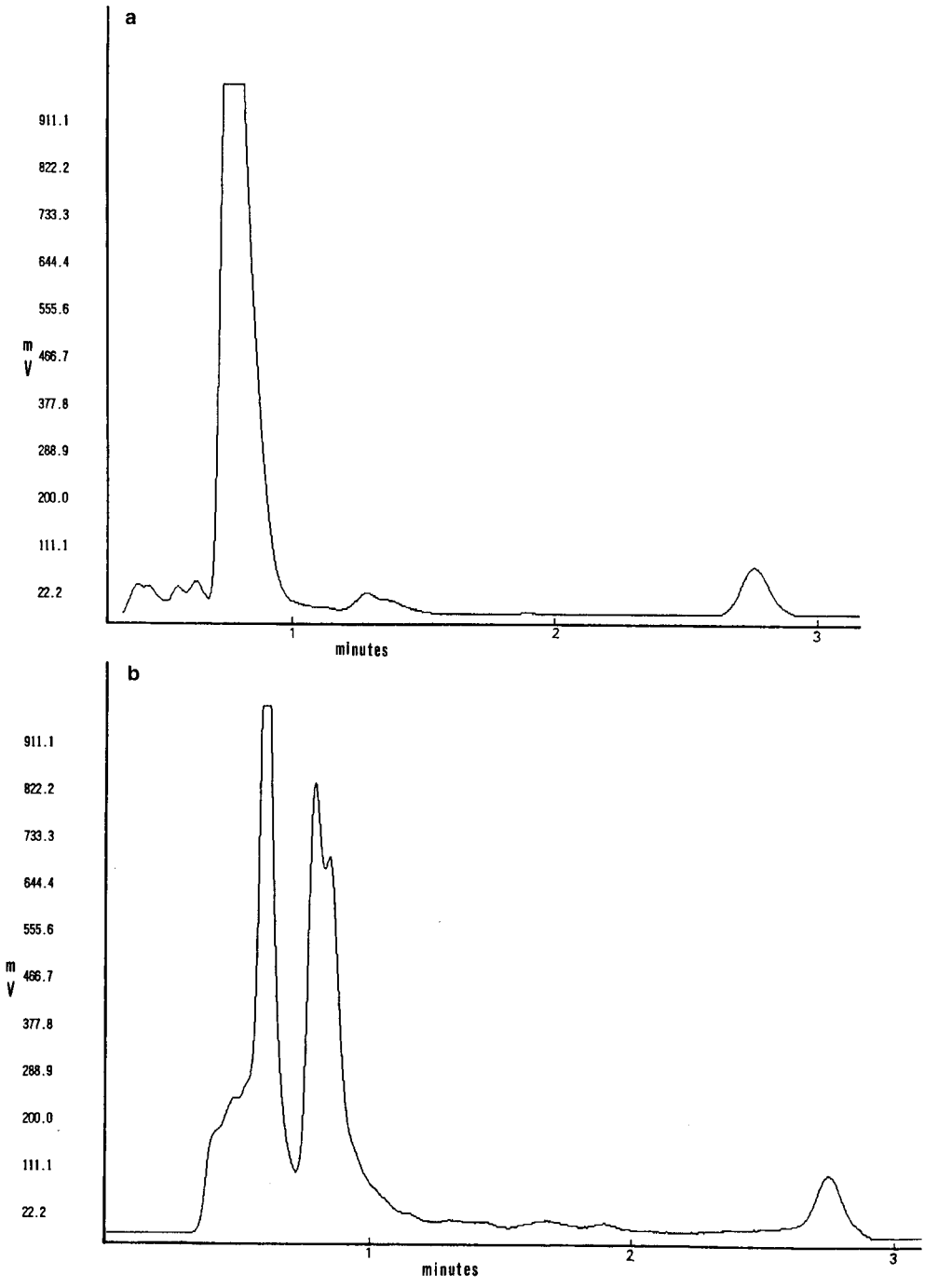

Fig. 1. HPLC of acetone DNPH derivative (a) in plasma and (b) in urine samples (retention time, 2 min 44 s).

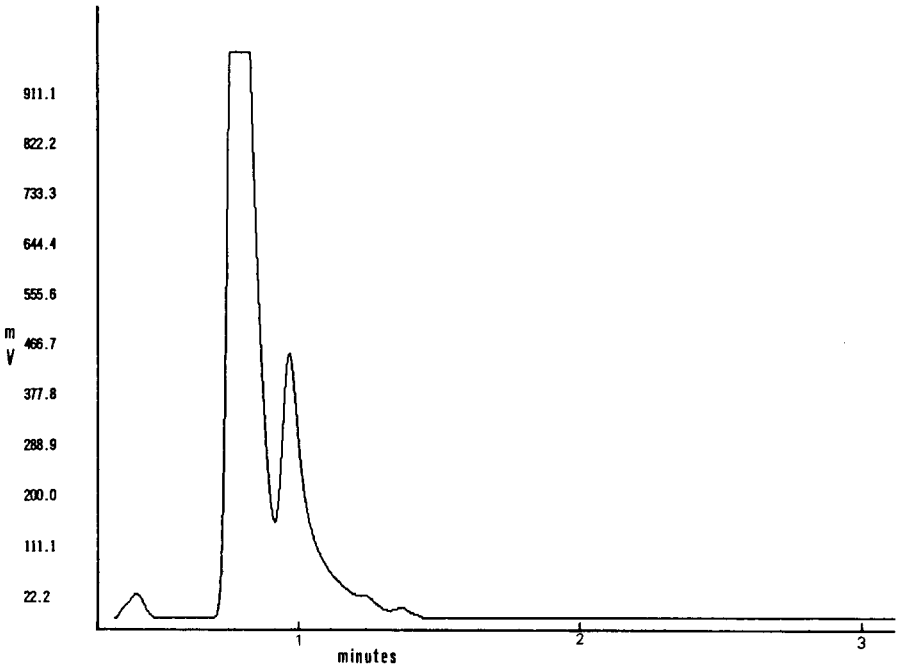

Fig. 2. HPLC of hydroxyacetone DNPH derivative (retention time, 57 s).

In the biological monitoring of workers exposed to acetone at the end of a shift and at the end of the working week, the acetone concentrations were 0.035–0.170 mmol/l with a mean value of 0.106 mmol/l in plasma and 0.103–1.29 mmol/l with a mean value of 0.320 mmol/l in urine. The determined concentrations were lower than the biological exposure indices given by different workers [22,24,25].

The range of acetone levels in plasma from two different classes of diabetic subjects (20 patients cured by diet with glucose values lower than 8.34 mmol/l and 30 patients with type I insulin-dependent diabetes mellitus on therapy with insulin) was 0.040–0.160 mmol/l with a mean value of 0.100 mmol/l.

DISCUSSION

The HPLC method described here overcomes many of the problems in the determination of acetone in biological fluids and the preanalytical errors. The volatile ketone is promptly stabilized by conversion into its DNPH derivative and rapidly determined without recourse to a solvent extraction step. If we wish to examine more samples in one chromatographic session, it is possible to minimize decarboxylation of acetoacetate and loss of acetone by preserving the samples at −20°C and thawing them at 4°C just before actone determination [14]. The added acetonitrile deproteinizes the plasma and simultaneously prevents the crystallization of the easily and rapidly formed DNPH derivative. The determination is free of interference from hydroxyacetone [26] and acetoacetate. The proposed method possesses the necessary sensitivity and shows excellent reproducibility. Up to 100 samples can be prepared in less than

30 min and individual chromatographic results obtained in less than 3 min. The method uses very inexpensive reagents. The proposed HPLC method can therefore be used to great advantage over current gas chromatographic methods; it can be used in experiments requiring multiple samples and specific activity determination for the routine measurement of acetone in diabetic patients and in biological monitoring of exposed workers. The use of very small sample amounts makes this a favourable method in paediatrics.

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Selective post-column fluorigenic reaction with benzamidine for trace level detection of reducing saccharides in liquid chromatography

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ABSTRACT

A liquid chromatographic post-column fluorigenic reaction with benzamidine for the trace level detection of reducing saccharides is described. The system consists of a Sugar Pak-1 gel column (300 mm × 6.5 mm I.D.) packed with a microparticulate resin in the calcium form and heated at 70°C. Elution is carried out with water containing 10^{-4} *M* CaEDTA at 0.4 ml min^{-1}. The post-column reaction is carried out with a mixture of 30 m*M* benzamidine and 1 *M* potassium hydroxide solution at 1 ml min^{-1} using a 530-μl reaction coil thermostated at 100°C. Fluorescence was monitored at 470 nm (emission) with an excitation wavelength of 360 nm. The system had good reproducibility (relative standard deviation 1–3%) for an injected amount of 0.9 μg (5 nmol). Linear calibration graphs were obtained between 16 and 500 nmol ml^{-1} ($r = 0.999$) and detection limits of 16–63 pmol at a signal-to-noise ratio of 3 were achieved. The detection of saccharides in plants is also described.

The separation and determination of saccharides is of considerable importance in diverse fields such as biology, biochemistry, medicine and food science. Liquid chromatography (LC) is the most commonly used technique for the analysis and purification [1] of simple monosaccharide mixtures and oligosaccharides. Different chromatographic techniques such as weak anion-exchange, reversed-phase and ligand-exchange modes have been used for this purpose and have been reviewed recently [2–5].

Detection of saccharides in the column effluent is commonly effected using either refractive index (RI) or ultraviolet (UV) detection. These types of detection are used when the analyte is present at a high concentration (micromole range) and if relatively little interfering material is present. RI detection is adequate for many classes of samples but the lack of selectivity and the low sensitivity of this detection system often require extensive sample clean-up. Further, strict control of the solvent composition (no elution gradients are possible) and of physical parameters (pressure, temperature and flow-rate) is necessary in order to avoid background noise. UV

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detection is generally carried out at 190 nm, but saccharides absorb only weakly at this wavelength and any impurities in the sample or the solvent with a strong chromophore may interfere. Consequently, UV detection has many of the same limitations as RI detection.

Another type of universal detector, based on mass detection, has been developed for carbohydrate analysis. This detector measures light scattering from a nebulized solute after solvent evaporation [6]. It can, in contrast to an RI detector, be used with solvent gradients but is not yet commonly found in most laboratories. Finally, in addition to these methods for direct detection, high-performance anion-exchange chromatography coupled with pulsed amperometric detection was developed in order to detect carbohydrates by electrochemical oxidation using a gold working electrode [7]. This method is more precise, sensitive (picomole range) and selective than RI detection.

The usefulness and sensitivity of conventional detectors based on UV absorption or fluorescence can be greatly increased by using pre- or post-column derivatization. For instance, pre-column derivatization with fluorigenic reagents (dansylhydrazine [8] or aminopyridine [9]) allows sensitive detection (10 pmol range) and is very useful for high-performance liquid chromatography (HPLC) on reversed-phase C_{18} or unmodified silica columns.

However, most studies have been performed with post-column reactors and many procedures have been published. Reducing sugars may be determined by post-column derivatization using a number of different fluorigenic reagents: 2-cyanoacetamide [10,11], ethanolamine [12], 2-aminoethylsulphonic acid (taurine) [13], 2-aminopropionitrile [14], arginine [15], ethylenediamine [16] and *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine [17]. These reagents react with reducing carbohydrates in an alkaline or neutral medium at 100–150°C to produce fluorescent derivatives; the advantages of these reagents have been described elsewhere [18]. With similar view, Kai *et al.* [19] recently developed a fluorimetric determination of reducing sugars by HPLC using *p*-methoxybenzamidine as a fluorigenic reagent in the post-column derivatization system.

Another aromatic amidine, benzamidine, can be used to determine reducing carbohydrates under alkaline conditions [20]. This compound has good selectivity for reducing saccharides in complex mixtures as it does not react with seventeen L-α-amino acids, aldehydes, keto-acids, carboxylic acids and nucleosides. Further, benzamidine reacts more rapidly with carbohydrates than other fluorigenic reagents, gives a higher fluorescence yield and is now commercially available.

The aims of this work were to study the use of benzamidine as a fluorigenic reagent for the detection of reducing saccharides such as D-glucose, D-galactose, D-fructose and lactose in a post-column derivatization system coupled to LC and the applicability of the proposed method to the determination of trace levels of sugars in plant extracts. In these natural matrices, monosaccharides are found at very low concentrations and interfering materials are present. Thus, sample preparation is necessary prior to the analysis in order to enrich and to clean up the sample.

EXPERIMENTAL

Chemicals

Benzamidine hydrochloride was obtained from Fluka (Buchs, Switzerland). All saccharides were obtained from Sigma (St. Louis, MO, U.S.A.). EDTA calcium disodium salt was obtained from Fluka and potassium hydroxide from Merck (Darmstadt, Germany). All aqueous solutions were prepared using doubly distilled, deionized water. The eluent was filtered and degassed in a Millipore filtration system (0.22 μm) before chromatographic analysis.

Apparatus

Chromatographic studies were performed with an LC-5000 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a Rheodyne 7125 six-way switching valve (50-μl loop). The column temperature was thermostated in a Varian CC1V Croco-cil column oven (Varian, Paris, France).

A Kontron (Zurich, Switzerland) Model 414-T pump was used for the post-column derivatization step.

Detection was performed by means of a Hitachi (Tokyo, Japan) Model F-1050 spectrofluorimeter. The fluorescence was monitored at excitation and emission maxima of 360 and 470 nm, respectively. Peak areas were calculated with a Hewlett-Packard (Palo Alto, CA, U.S.A.) Integrator Model 3380-A.

Procedure

Separations were carried out using a 300 × 6.5 mm I.D. gel column packed with a microparticulate resin in the calcium form (Waters–Millipore, Milford, MA, U.S.A.) and thermostated at 70°C. The aqueous mobile phase contained 10^{-4} *M* CaEDTA to regenerate calcium in the resin and was pumped at 0.4 ml min^{-1}. The separation of saccharides was achieved by a ligand-exchange mechanism on the column packed with a polymer-based cation-exchange material (sulphonated polystyrene–divinylbenzene). Unlike the elution order on silica-based columns, saccharides elute as a function of decreasing degree of polymerization. This effect is ascribed to a size-exclusion mechanism, which is assumed to take place in addition to a ligand-exchange effect. Calcium-loaded columns allow the separation of mono- and disaccharides and sugar alcohols with good selectivity. Such columns are also compatible with the presence of inorganic anions in the eluent or in the sample, and may be readily regenerated.

Post-column reaction conditions

The reagent was prepared by adding a 30 m*M* aqueous benzamidine solution to a 1.0 *M* solution of potassium hydroxide. A 530-μl reaction coil (PCR1; Varian) thermostated at 100°C was used for the post-column reaction. The post-column reagent flow-rate was 1.0 ml min^{-1}.

Sample pretreatment

Adult vegetative plants of *Sinapis alba* L. (mustard) were used. This material was used as the environmental conditions that induce flowering of the plant also change the concentration of the reducing carbohydrates present [21]. The tissues were

sealed in glass tubes containing 10 ml of 80% ethanol in water per gram fresh weight. The tubes were then heated at 100°C for 15–30 min in a water-bath. Preliminary experiments showed that this method allows a complete extraction of glucose and fructose [21]. The extract was evaporated to dryness and the residue dissolved in distilled water.

Because of the presence of interfering materials in the aqueous extract, the following sample clean-up procedure was used. Solid-phase extraction columns (Analytichem International; trimethylaminopropylsilica SAX and cyclohexylsilica CH) were activated by washing with 4 ml of methanol and 4 ml of water. As carbohydrates are neither anionic nor non-polar, they pass through both sorbents unretained. Organic acids are too polar to be retained on the CH sorbent and so they pass onto the SAX, where they are retained. The phenols are retained on the non-polar CH sorbent. Following this clean-up procedure, a 50-μl aliquot of the carbohydrate extract was injected into the chromatograph.

RESULTS AND DISCUSSION

Optimization of the post-column reaction system

The conditions used for the separation and post-column derivatization were investigated by using four reducing saccharides: D-glucose, D-fructose, lactose and D-galactose. Reducing sugars were separated on a gel column packed with a microparticulate resin in the calcium form with water as the mobile phase. CaEDTA, used in the eluent to regenerate calcium on the resin, does not interfere with the post-column derivatization. The use of ligand exchange is an advantage in this separation system including a chemical post-column reaction as an aqueous effluent can be used.

It has been suggested [22] that the post-column derivatization reaction between benzamidine and reducing sugars involves the coupling of the keto alcoholic moiety of carbohydrates to the amidino group of benzamidine. This reaction is carried out in concentrated potassium hydroxide and yields a fluorescent imidazole derivative [22].

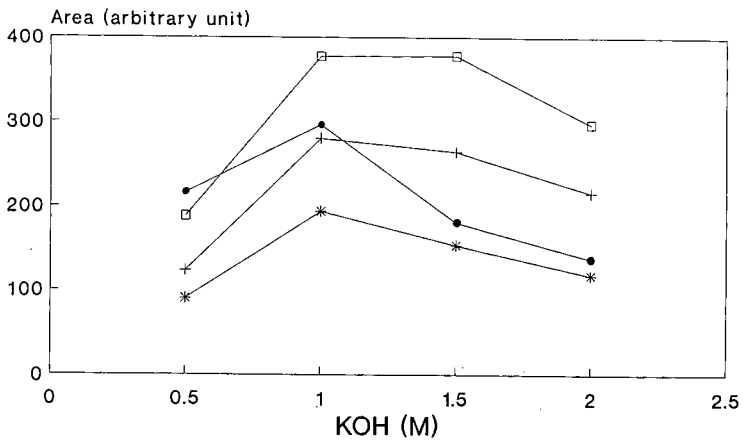

Fig. 1. Influence of potassium hydroxide concentration on the peak areas of four reducing carbohydrates. ● = Lactose; + = D-glucose; * = D-galactose; □ = D-fructose.

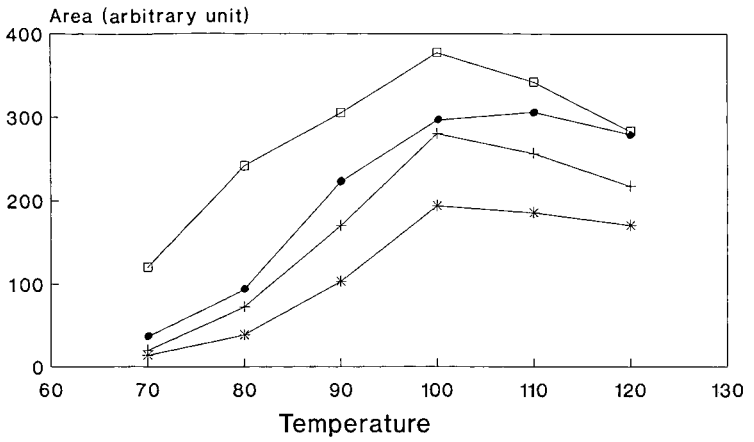

Fig. 2. Influence of reaction temperature (in °C) on the peak areas of four reducing carbohydrates. Symbols as in Fig. 1.

As potassium hydroxide is mixed with the column effluent, an important parameter to optimize is the concentration of this reagent in order to obtain the maximum fluorescence intensity. In Fig. 1, the influence of the concentration of potassium hydroxide is shown for four different saccharides. Maximum intensity was obtained using 1.0 *M* potassium hydroxide for all the reducing carbohydrates tested. D-Fructose was found to give the highest and D-galactose the lowest fluorescence intensity.

We also tested the influence of reaction temperature, as shown in Fig. 2. Optimum fluorescence was obtained at 100°C. Again, D-galactose shows the weakest response. It is important to note that even at lower temperatures, a good response can be obtained with ketohexoses such as D-fructose whereas only a very weak signal was obtained with aldohexoses.

Based on the above considerations, post-column derivatization of reducing sugars with benzamidine is best carried out at 100°C and in 1.0 *M* potassium hydroxide solution. These conditions were therefore used for all the experiments reported in this paper. The reaction time in chemical reaction detectors is determined by the flow-rates of the column effluent and the fluorigenic reagent and by the dimensions of the reactor. In our experience, a reaction time of *ca.* 20 s is sufficient.

Chromatographic analysis

Many reducing carbohydrates other than D-glucose, D-galactose, D-fructose and lactose were tested under the recommended conditions. The retention times for these saccharides and their respective detection limits are given in Table I. The retention times decreased with increasing column temperature up to 90°C, the recommended temperature. The column was kept at 70°C in this system in order to obtain a better separation of the different carbohydrates. As can be seen, possible coelution problems could exist between L-rhamnose, D-xylose, D-galactose and D-mannose (12.16–12.53 min) and between D-fructose and L-fucose (13.48–13.69 min). If separation of these compounds is necessary (which was not the case in our study), it would be necessary to modify the composition of the mobile phase.

TABLE I

RETENTION TIMES AND LIMITS OF DETECTION (LOD) FOR VARIOUS REDUCING CARBOHYDRATES

LOD is defined as the amount in a 50-μl injection volume to give a signal-to-noise ratio of 3.

Carbohydrate	Retention time (min)	LOD (pmol)
Lactose	8.94	20.8
D-Glucose	10.92	15.8
L-Rhamnose	12.16	62.5
D-Xylose	12.16	41.7
D-Galactose	12.25	20.8
D-Mannose	12.53	62.5
D-Fructose	13.48	15.8
L-Fucose	13.69	62.5
L-Arabinose	13.99	41.7

Detection limits, based on a 3-fold signal-to-noise ratio at the baseline, ranged from 16 to 63 pmol for a 50-μl injection. The precision was established by repeated determinations ($n=6$) of a standard mixture of the nine reducing carbohydrates for an injected amount of 5 nmol. The relative standard deviations (R.S.D.s) were D-glucose 1.3, lactose 1.9, D-galactose 2.8, D-fructose 2.0, L-rhamnose 2.4, L-arabinose 1.7, D-xylose 2.7, L-fucose 1.4 and D-mannose 3.2%.

A typical chromatogram of a standard mixture of four reducing carbohydrates, obtained by ligand-exchange HPLC with post-column derivatization using benzamidine, is shown in Fig. 3. The fluorescence excitation and emission maxima of the final reaction mixtures of all the carbohydrates were 360 and 470 nm, respectively, and the fluorescence spectra were almost identical with those obtained in the manual spectrofluorimetric method [20].

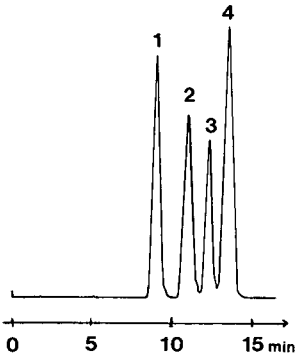

Fig. 3. Chromatogram of a standard mixture of four reducing carbohydrates. Peaks: 1 = lactose; 2 = D-glucose; 3 = D-galactose; 4 = D-fructose. Concentration, 10^{-4} *M*; column, 300 × 6.5 mm I.D.; stationary phase, Ca^{2+} ligand-exchange; column temperature, 70°C; mobile phase, 10^{-4} *M* aqueous CaEDTA; fluorimetric detection, λ_{ex} = 360 nm, λ_{em} = 470 nm; sensitivity, 0.012 a.u.f.s.; flow-rate, 1.4 ml min^{-1}.

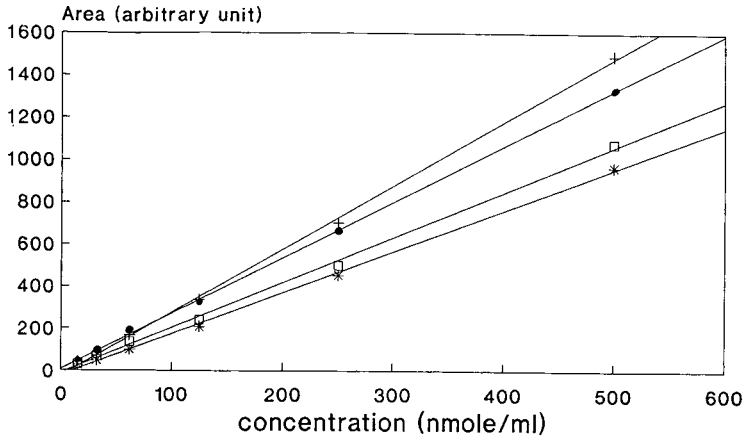

Fig. 4. Calibration graphs for HPLC of reducing carbohydrates. Aliquots (50 μl) of sugar standards were analysed as described in the text. Each point in the mean of six determinations. ● = D-Glucose; + = D-fructose; * = lactose; □ = D-galactose.

Calibration graphs, obtained by plotting peak area *versus* the concentration of four reducing carbohydrates, are displayed in Fig. 4. They were linear over the concentration range 16–500 nmol ml^{-1} ($r = 0.999$) and passed through the origin.

Peak broadening due to the post-column derivatization system was tested by injecting D-fructose with and without the post-column reactor being installed. It was calculated by means of the Foley and Dorsey [23] experimental approximation of the standard deviation (σ), based on the measurement of peak width and peak asymmetry at 10% of the peak height. The 530-μl reactor caused an additional peak broadening

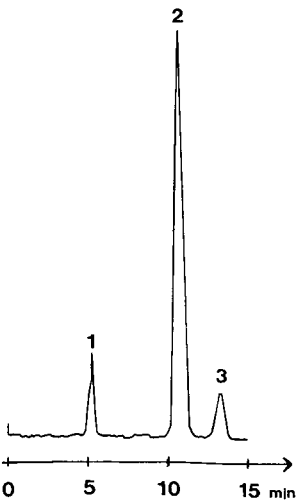

Fig. 5. Chromatogram of plant sample No. 2 (see Table II). Peaks: 1 = unidentified compound; 2 = D-glucose; 3 = D-fructose. Conditions as in Fig. 4. Sensitivity: 0.05 a.u.f.s.

TABLE II

DETERMINATION OF D-FRUCTOSE AND D-GLUCOSE IN MUSTARD EXTRACTS

Sample[a]	n	D-Fructose		D-Glucose	
		Mean concentration (μM)	R.S.D. (%)	Mean concentration (μM)	R.S.D. (%)
1	3	47	2.4	210	3.2
2	5	26	1.0	192	3.9
3	5	28	1.2	242	3.1

[a] 1, 2 and 3 represent different samples.

of $\sigma = 4.0$ s, which is relatively small compared with the peak broadening of the total chromatographic system (without reactor) of $\sigma = 15.5$ s.

Following these results, benzamidine proved to be easy to use as a fluorigenic reagent and to be compatible with on-line coupling to LC analysis of reducing carbohydrates. Indeed, the reaction time, the temperature and the addition of 1 M potassium hydroxide can be performed easily in a post-column reacting coil. Further, benzamidine is commercially available. Other fluorigenic derivatization agents have been developed by different workers (see Introduction); the aim of this work was not to compare these reagents but to develop a simple, sensitive and selective method for reducing carbohydrates. Benzamidine satisfies all these requirements.

Application to carbohydrate determination in plant samples

In order to apply the above method to a natural sample, the post-column derivatization system was used to determine monosaccharides in plant extracts. The complexity of the biological matrices and the low concentrations of monosaccharides led us to develop a very selective and sensitive method. Fig. 5 shows a typical chromatogram obtained with a plant extract. Aqueous sugar standards were pretreated in the same way as the samples and were used to prepare a calibration graph.

The sugar contents of plant extracts are summarized in Table II. The results were comparable to those given in the literature [21] and the differences fell within experimental error. This suggests that possible matrix effects were not significant and that the benzamidine post-derivatization technique has a good selectivity for reducing sugars in complex biological samples. The R.S.D.s of this post-column method for D-glucose and D-fructose are given in Table II and varied between 1 and 4%. The results show that this method is very selective for the study of reducing carbohydrates in plant samples. Such studies are of growing importance for process technology.

CONCLUSION

The post-column derivatization procedure using benzamidine as a fluorigenic reagent allows the LC determination of reducing sugars at the picomole level. This combination uses a ligand-exchange LC column for the separation of reducing carbohydrates and is more rapid, precise, sensitive and selective than LC with RI detection. The system described demonstrated high selectivity for the determination of several

carbohydrates in plant materials. Consequently, the method could be readily used for screening purposes, *e.g.*, to check disorders of carbohydrate metabolism, to study plant metabolism or for the characterization of wines through their fermentable sugar fingerprint. Future research will include some of these aspects and validation of the proposed method for these different media. In particular, the analytical results for reducing sugars in different plant materials will be compared by both a batch enzymatic test and the proposed post-column derivatization method.

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Determination of free DOPA and 3-O-methyl-DOPA in human plasma by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A procedure was devised for the determination of the unconjugated non-protein-bound fraction of 3,4-dihydroxyphenylalanine (DOPA) and 3-O-methyl-DOPA (3-OMD) in plasma using a reversed-phase liquid chromatographic system coupled with electrochemical detection. Sample preparation involves rapid isolation of the unbound drugs from the drug–protein complex by ultrafiltration through a membrane with a molecular weight cut-off of 10 000 dalton. One chromatographic run requires less than 10 min. The relative standard deviation is < 3% for the within-assay imprecision and < 4% for the between-assay imprecision. The detection limits for DOPA and 3-OMD are 0.2 and 1.3 ng/ml, respectively.

INTRODUCTION

Treatment of Parkinson's disease is still based on substitution therapy with 3,4-dihydroxyphenylalanine (DOPA), alone or in combination with a peripheral decarboxylase inhibitor, in spite of the side-effects that may arise (dyskinesia, on–off phenomenon and psychoses), particularly during long-term therapy [1,2]. Elevated concentrations of 3-O-methyl-DOPA (3-OMD) have been associated with DOPA-induced side-effects [3,4].

For a more complete understanding of the mechanism of action of this drug, we consider that its plasma concentration together with that of its metabolite should be monitored in order to establish the amount that could cross the blood–brain barrier. In addition, it is important to assess whether DOPA and 3-OMD bind significantly to plasma proteins. In fact, it is generally accepted that only the free (non-protein-bound) fraction of drugs is available to equilibrate with receptor sites in tissues [5], even though with DOPA the existence of an active uptake system in the blood–brain barrier [6] may represent a complicating factor. Papers dealing with methods for determining DOPA and 3-OMD in plasma by high-performance liquid chromatography (HPLC) with electrochemical detection have been published [7–10], but none has taken into account their unconjugated free (non-protein-bound) fraction.

This paper describes a micro-scale method for the determination of free DOPA

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and 3-OMD not protein bound in plasma; no sample pretreatment other than ultrafiltration is needed.

EXPERIMENTAL

Reagents and standards

DOPA and 3-OMD were purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and water were of HPLC grade (Merck, Darmstadt, Germany). All other chemicals were of analytical-reagent grade.

The elution buffer was composed of a solution of 50 mmol/l sodium dodecyl sulphate and 100 mg/l ethylenediaminetetraacetic acid disodium salt adjusted to pH 2.5 with 2 *M* phosphoric acid.

Stock solutions of DOPA and 3-OMD were prepared at 1 mg/ml in 0.1 *M* perchloric acid. Working standard solutions of 20, 40 and 80 ng/ml DOPA and 0.5, 1.0 and 2.0 μg/ml 3-OMD were prepared by serial dilutions with filtered isotonic saline. The stock standard solutions were stored at −20°C and working solutions were prepared every day. A 500-μl volume of the working standard solutions was treated as if it was a specimen from a patient.

Apparatus and chromatography

The HPLC system consisted of a Model 1350 pump (Bio-Rad Labs., Richmond, CA, U.S.A.) equipped with a Rheodyne Model 7125S injection valve fitted with a 20-μl sample loop. The chromatographic column (150 mm × 4.6 mm I.D.) was packed with Rosil C_{18} HL, particle size 3 μm (Bio-Rad Labs.). A cartridge head with interchangeable cartridges (40 mm × 4.6 mm I.D.) packed with ODS-5S (particle size 5 μm) (Bio-Rad Labs.) was screwed onto the column head. The electrochemical detection system (Coulochem 5100 A; ESA, Bedford, MA, U.S.A.) consisted of a conditioning cell (Model 5021) and an analytical cell (Model 5010) containing dual coulometric electrodes. The overall system operated in the redox mode. The conditioning cell was set at +0.30 V. The working potentials of the two electrodes of the analytical cell were 0.00 for the first and −0.30 V for the second. Signals from the detector were converted to a chromatographic trace by a Bio-Rad Labs. Model 1322 recorder. The mobile phase was elution buffer–acetonitrile (88:12), filtered through a 0.22-μm membrane filter (GS type; Millipore, Molsheim, France). Isocratic elution was carried out at room temperature at a flow-rate of 1.0 ml/min.

Procedure

Blood samples from a patients receiving DOPA were drawn by venipuncture, transferred into heparinized tubes and centrifuged at 3000 *g* for 10 min at 4°C. Plasma was immediately separated and 500 μl were centrifuged at 3000 *g* for 30 min through a membrane Minicent 10 (Bio-Rad Labs.), which restricts the passage of species larger than 10 000 dalton. Then 20 μl of the ultrafiltrate were injected directly into the chromatographic system. Samples were eluted isocratically and quantified by comparing the height of the peaks with those of standard solutions that had also been ultrafiltered. When operating for more than 24 h, 100 μl methanol should be injected into the column instead of the sample. This eliminates the lipids that have coated the electrodes and would be responsible for shifting the potential of the reference and auxiliary electrodes.

RESULTS AND DISCUSSION

The most delicate aspect of this procedure was to obtain a plasma free from protein without using either aggressive reagents, such as acids, or organic solvents, as both break the drug–protein bonds. To collect plasma free from proteins we suggest using an ultrafiltration membrane that has a molecular weight cut-off of 10 000 dalton. With the present procedure we obtained 200 μl of ultrafiltrate by centrifuging 500 μl of plasma for 30 min at 3000 g. The potentials of the three electrodes were selected after injection of fixed amounts of the standards. The choice of +0.30, 0.00 and −0.30 V, similar to those used by Baruzzi *et al.* [10], was a good compromise between high sensitivity and low noise. In fact, the use of a higher potential to oxidize 3-OMD completely gives rise to a significant increase in the baseline noise [9].

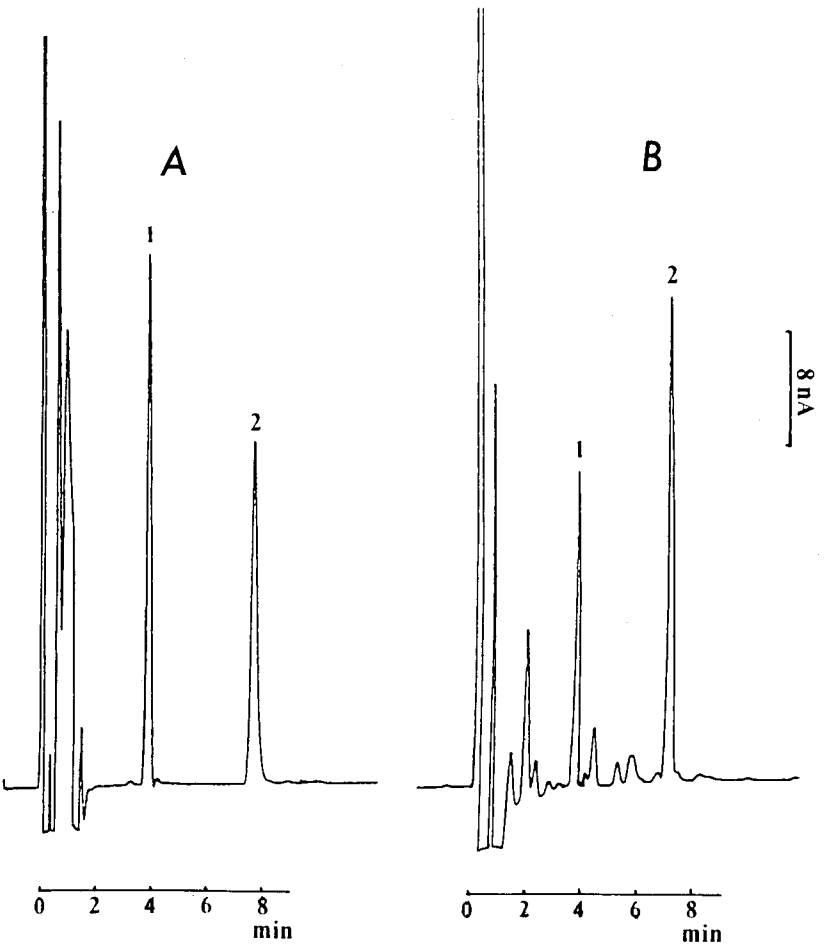

Fig. 1. Separation of (1) DOPA and (2) 3-OMD in (A) a standard solution of the compound and (B) an ultrafiltered plasma sample. The standard solution contained 20 ng/ml of DOPA and 1.0 μg/ml of 3-OMD. The plasma sample contained 11.7 ng/ml of DOPA and 1.42 μg/ml of 3-OMD.

A representative chromatogram showing the separation of a standard mixture of DOPA and 3-OMD is shown in Fig. 1A. Fig. 1B is the chromatogram of a plasma sample, prepared as described above, from a patient receiving DOPA. The separation and elution of the two compounds of interest were completed in 10 min.

From the analysis of ultrafiltered plasma, the identity of the chromatographic peaks is demonstrated in several ways. First, there is complete correspondence between the retention times of the samples and those of the standards. Second, on increasing the concentration of acetonitrile in the mobile phase, the retention times for standards and samples are markedly altered in the same manner. Third, the ratio of the peak areas of the standards and the samples measured at differed potentials is the same (signature) [11].

Other principal metabolites of DOPA, such as 3,4-dihydroxyphenylacetic acid (DOPAC), hydroxymethylmandelic acid (HMMA) and homovanillic acid (HVA), did not interfere in the separation of DOPA and 3-OMD. Dopamine, which arises after decarboxylation of DOPA, did not be interfere in the assay under the conditions described.

In order to investigate whether DOPA and 3-OMD bind to the filter membrane, we compared both the working standard solutions and the plasma samples deproteinized with 1.2 *M* perchloric acid [10] both before and after ultrafiltration. In the ultrafiltrate we found values ranging from 97% to 102% for both substances, showing that the membrane of the ultrafilter does not seem to bind at any level considered for DOPA and 3-OMD.

Linearity was assessed from injection of DOPA, 3-OMD standard mixtures in the concentration range 0.2–1000 ng/ml for DOPA and 1.0–5000 ng/ml for 3-OMD. For both substances a linear relationship between the amount injected and the detector signal was demonstrated. Under the assay conditions, the detection limits were 0.2 and 1.3 ng/ml for DOPA and 3-OMD, respectively, at a signal-to-noise ratio of 2.

To determine the reproducibility of the assay, a plasma from a patient receiving DOPA (125 mg, three times a day) was analysed ten times in the same run. The relative standard deviation (R.S.D.) was 2.5% for DOPA at a concentration of 106 ng/ml and 2.1% for 3-OMD at a concentration of 1.5 μg/ml. Analyses of the same sample over a 10-day span (the sample was stored in aliquots at −20°C between assays) yielded an R.S.D. of 3.4% for DOPA and 3.6% for 3-OMD.

In the plasma samples collected from four Parkinsonian patients (assuming doses of 125 mg of DOPA and 12.5 of carbidopa, three times a day) containing total DOPA ranging from 260 to 390 mg/ml, the free fraction was between 8% and 13% of the total concentration. In the same samples the total 3-OMD ranged from 0.90 to 1.55 μg/ml and the free fraction was between 18% and 21% of the total concentration.

In conclusion, the specific and sensitive method described may offer a means of determining the free unconjugated fractions of DOPA and its metabolite 3-OMD. The method is characterized by high recovery and good reproducibility; it is well suited for routine operation with extensive test runs.

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High-performance liquid chromatographic determination of sugars and polyols in extracts of lichens and sugarcane juice

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ABSTRACT

Several polyols and monosaccharides can be efficiently separated from standard mixtures by high-performance liquid chromatography. Separation is carried out under isocratic conditions using acetonitrile–water (80:20, v/v) as mobile phase. Detection is performed by measurement of UV absorbance at 195 nm, which improves the sensitivity of the method. Linearity for both polyols and sugars is obtained over a wide range of concentrations. The method was applied to the determination of the polyol content of two lichen extracts, obtained from *Evernia prunastri* and *Himantormia lugubris*. Xylose, arabinose and galactose were identified, for the first time, in crude juices from sugarcane, in addition to the well known fructose, glucose and sucrose. Two main fractions of soluble polysaccharides could also be separated from the same juice and analysed, after acidic hydrolysis, as being composed of fructose and galactitol.

INTRODUCTION

The assessment of the carbohydrate and polyhydric alcohol content of plants, and the biotrophic transport of these products, is important in the analysis of several physiological processes. Many C3 [1] and several C4 grasses [29], such as sugarcane, produce fructans when sucrose biosynthesis and transport exceed the physiological demand. Biological production of fructans involves two main enzyme systems, sucrose–sucrose–fructosyl transferase (SST) and fructan–fructan–fructosyl transferase (FFT) [3], the products of which have sometimes been determined by high-performance liquid chromatography (HPLC). In contrast, hydrolysis products of fructans, achieved by several fructanases, are commonly detected by thin-layer or gas–liquid chromatography (GLC) [4]. These procedures are often used to analyse the sugar composition of plant extracts, such as sugarcane juices, in which only glucose and fructose appear as free monosaccharides [5].

Polyols are produced by many plant species but are predominantly accumulated in lichens [6]. A high proportion of all the carbon fixed by the algal partner passes to the fungus as a single type of molecule. This molecule is ribitol in lichens containing a green alga as photobiont or glucose in cyanobiont-containing lichens [7]. Moreover, studies using intact thalli show that most of the mobile carbohydrates are immediately converted to fungal polyols, especially mannitol and arabitol [8].

Several methods have been developed for studying carbohydrates and polyols

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and applied to several plant preparations. HPLC was first applied to the study of lichen metabolites to elucidate the derivatives of the acetate–polymalonate pathway [9]. Gordy *et al.* [10] developed an HPLC method for application to carbohydrates and polyols from lichens, using Bondapak and Aminex Q-150 S columns. MacFarlane and Kershaw [11] separated carbohydrates from several lichen species by using a Bio-Rad Labs. Aminex HPX-87C column. Similar methods have been applied to identify sucrose, glucose and fructose in sugarcane juices [5,12] and sugars produced after polysaccharide hydrolysis in many grasses. A refractive index detector, compatible only with isocratic elution, was always used.

In this paper, we report the determination of polyols and sugars by HPLC using a UV detector at 195 nm instead of a refractive index detector, improving the sensitivity of sugar determination.

EXPERIMENTAL

Plant material

Saccharum officinarum L., field-grown, *Evernia prunastri* (L.) Ach., growing on branches of *Quercus pyrenaica* Willd., and *Himantormia lugubris* (Hue) Lamb, growing on soil in King George Island (Antarctica), were used.

Sample preparation from sugarcane stalks

Stems of 17 month-old plants were mechanically crushed and the crude juice produced was adjusted to pH 8.0 by adding saturated ammonium carbonate solution. The juice was then centrifuged at 20 000 *g* for 15 min at room temperature and the supernatant was filtered through Whatman No. 3 filter-paper. Sodium azide was added to the filtrate to reach a final concentration of 0.02% (w/v). A 10-ml aliquot of this clarified juice was then filtered through a Sephadex G-10 column (15 cm × 2.5 cm I.D.), equilibrated at pH 8.0 with aqueous ammonium carbonate solution containing 0.02% sodium azide.

Calibration of this column was performed by filtering through it 5.0 ml of 0.02% (w/v) blue dextran 2000 and 10% (w/v) cobalt(II) chloride solution. Elution was carried out with distilled water. Fractions (1.0 ml) 1–20 were discarded. Fractions 21–32 ml were collected and stored to prepare a heterogenous mixture of fructans [13]. Low-molecular-weight sugars, including sucrose, were eluted from the Sephadex G-10 column from 42 to 58 ml of filtrate [14] and detected by the method of Dubois *et al.* [15]. This fraction was lyophilized and stored at −38°C. When required, residues were dissolved in 2.5 ml of acetonitrile–water (80:20, v/v) and loaded onto the chromatographic column. Fractions 20–32 ml were filtered through a Sephadex G-50 column (30 cm × 2.5 cm I.D.), equilibrated as above. Fractions 40–70 ml from this last column contained soluble polysaccharides (SP preparation), whereas mid-molecular-weight carbohydrates (MMWC preparation) eluted in fractions 70–120 ml.

Extraction of sugars and polyols from thalli of E. prunastri and H. lugubris

Air-dried thallus samples (2.0 g) were rehydrated at room temperature for 5 min with distilled water, gently dried with filter-paper and macerated with 20 ml of acetone to remove lichen phenols [16]. The dry residues were then ground with 10 ml of cold 80% ethanol, filtered through a double cheese-cloth and the filtrates stored at

− 13°C for 14 h. The precipitates were then discarded and the supernatants heated at 60°C for 20 min. To an aliquot (5 ml) of clear supernatant, 5 ml of 80% cold ethanol were added and then heated again to dryness. This procedure was repeated three times under the same conditions as above. The last residues were reconstituted with 5.0 ml of cold 80% ethanol and centrifuged at 3000 *g* for 15 min. The supernatants were evaporated to dryness under reduced pressure [17]. Each residue was dissolved in 2.5 ml of acetonitrile–water (80:20, v/v); and loaded into the chromatographic column.

Reagents

Polyols (ribitol, arabitol, galactitol and mannitol) and sugars (D-xylose, D-arabinose, D-ribose, D-fructose, D-galactose, D-glucose, D-mannose, D-rhamnose and sucrose) were provided from Sigma (St. Louis, MO, U.S.A.). Acetonitrile (HPLC grade) (Carlo Erba, Milan, Italy) was used as received and doubly distilled water (Carlo Erba) was filtered through Millipore GS filters (0.22-μm pore diameter) before use.

A Micropack NH_2 column (Varian, Palo Alto, CA, U.S.A.), supplied in hexane for use in normal-phase chromatography, was prepared for sugar analysis employing the following series of solvents: 20 ml of isopropanol at a flow-rate of 1.0 ml min^{-1}, 20 ml of distilled water at 1.0 ml min^{-1}, 60 ml of 0.1 *M* H_3PO_4 (pH 1.5) at 2.0 ml min^{-1}. The column was finally washed with pure acetonitrile and kept in this solvent until required.

HPLC separation of sugars and polyols

HPLC was performed on a Varian Model 5060 liquid chromatograph equipped with a Varichrom TM VUV/10 UV detector and a Vista CDS 401 computer. The chromatographic conditions were as follows: column, MicroPack NH_2 10 P/N (30 cm × 3 mm I.D.) from Varian; sample loading, 10 μ; mobile phase, acetonitrile–water (80:20, v/v) isocratically or with a gradient where indicated; flow-rate 1.3 ml min^{-1}; temperature, 20°C; detector, UV (195 nm), 0.005 a.u.f.s.; attenuation, 16; internal standard, 2.0 mg ml^{-1} D-ribose when polyols were analysed and 2.0 mg ml^{-1} ribitol when sugars were analysed.

RESULTS

Separation of polyols

The main problem in the separation and determination of polyols from lichens is the difficulty of a clear resolution between enantiomers. In this work, the complete separation of the enantiomers ribitol and arabitol, and also mannitol, was achieved using a MicroPak NH_2 10 P/N column, packed with 10-μm silica which has a chemically bonded phase containing aminopropyl groups. The polar alkylamine stationary phase, which has strong hydrogen-bonding properties, separates sugars by a partition mechanism. Reconversion of the normal-phase column by the use of the solvent series described above permits this column to be used in the reversed-phase mode. This allows the use of acetonitrile as a component of the mobile phase, which improves sugar separations by decreasing the polarity of the mobile phase and, as a consequence, increasing the sugar retention.

Complications arise from the use of the detector at 195 nm, as there is a slight baseline shift for several reasons. The use of a wavelength of 195 nm, 0.005 a.u.f.s. and acetonitrile–water as the mobile phase implies very extreme conditions of analysis. This also produces a large background absorbance. Therefore, even with an isocratic procedure, baseline correction must be applied. This is constructed from the start of the first peak to the lowest valley point at the end of the same peak and, in this way, after a baseline segment has been constructed, the area of the peak is corrected. The result is stored in a time and area file and then the next baseline segment is calculated for the included peaks. Each successive baseline segment starts at the end of the preceding one, to include all the peaks. A signal-to-noise ratio of 1.0 is included in the program for the method, which allows the detection of very small positive peaks representing sugar alcohols. Under these conditions, stability of the response is higher than that obtained by using only water as solvent for separating sugars in the normal-phase mode.

Analysis for ribitol, arabitol and mannitol is complete in less than 13 min, although the complete elution procedure for the next injection needs about 30 min (Fig. 1). The calibration graphs shown in Fig. 2 indicate that the goodness of fit is

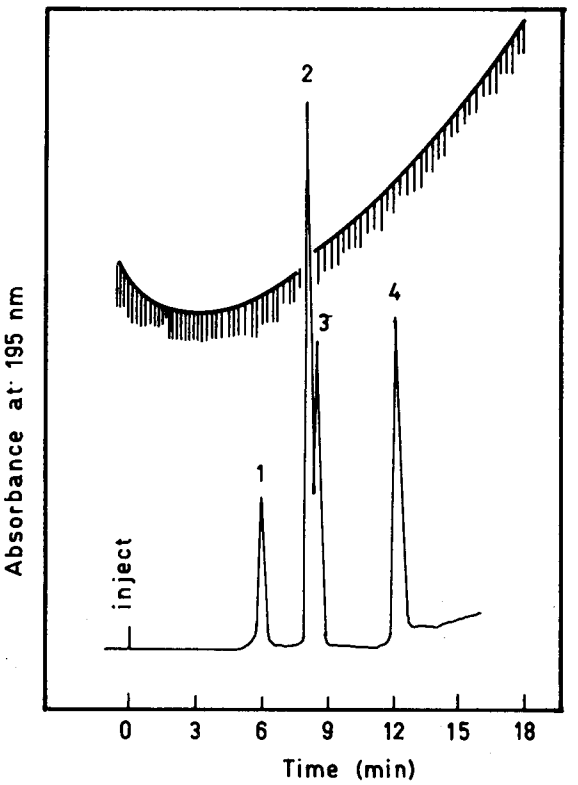

Fig. 1. HPLC separation of a standard mixture of 10 μg each of D-ribose, ribitol, arabitol and mannitol on a MicroPak NH_2-10 P/N column. Solvent, acetonitrile–water (80:20, v/v), isocratic. Flow-rate, 1.3 ml min^{-1}. Peaks: 1 = D-ribose as internal standard; 2 = ribitol; 3 = arabitol; 4 = mannitol. The line drawn across the chromatogram indicates baseline correction.

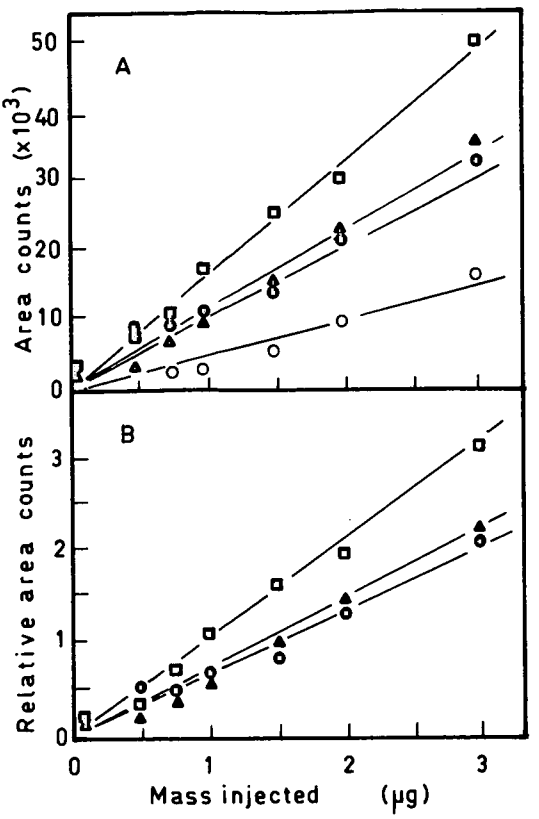

Fig. 2. Calibration lines of sugar alcohols by HPLC. Chromatographic conditions as in Fig. 1. (A) Direct calibration of (○) D-ribose, $y = 6.10x - 3.24$, $r^2 = 0.99$; (●) ribitol; $y = 10.36x + 0.15$, $r^2 = 0.99$; (▲) arabitol, $y = 11.59x - 1.04$; $r^2 = 0.99$; and (□) mannitol, $y = 11.85x - 0.006$, $r^2 = 0.99$. (B) Calibration with respect to an internal standard (2.0 mg ml^{-1} D-ribose) of (●) ribitol, $y = 0.67x + 0.008$, $r^2 = 0.99$, (▲) arabitol, $y = 0.75x - 0.07$, $r^2 = 0.99$ and (□) mannitol, $y = 1.03x - 0.005$, $r^2 = 0.99$. Data are the means of four replicates. The standard error was never larger than the symbols.

almost perfect and that the method is linear from at least 1.0 to 3.0 μg of each polyol injected. Equations fitted by linear regression have a determination coefficient of about 0.99 in all instances.

Gradient elution is often used to decrease the separation time and sharpen peaks, but adds complexity to the separation because of the column equilibration time and baseline offset, especially with low wavelength detection. Fig. 3 shows the chromatographic trace obtained by applying a linear gradient from 80:20 to 60:40 (v/v) acetonitrile–water in 30 min. The retention times of the three polyols decrease by *ca.* 0.4 min. Mannitol elutes 1.5 min earlier when gradient instead of isocratic elution is used.

The plate number (N) is *ca.* 4000. Maximum N values for ribitol (3850) and arabitol (4220) are obtained with an isocratic composition of the mobile phase, although the maximum plate number for mannitol (3823) is achieved with gradient

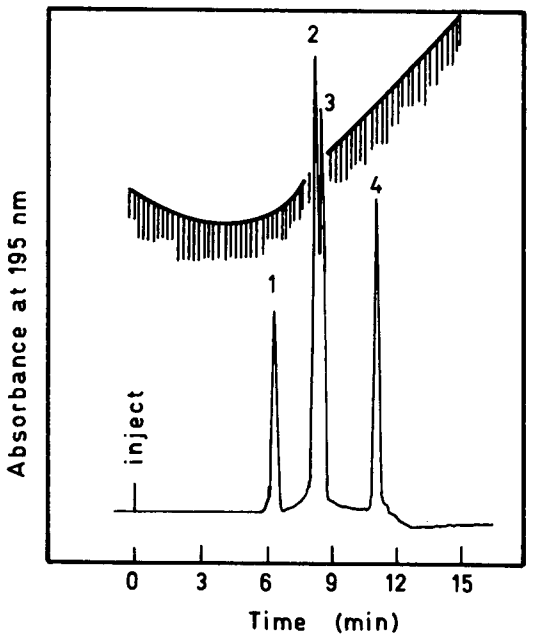

Fig. 3. HPLC separation of a mixture of 10 μg each of D-ribose, ribitol, arabitol and mannitol on the same column as in Fig. 1. The mixture was eluted from the column at 1.3 ml min^{-1} with a linear gradient from 80:20 to 60:40 (v/v) acetonitrile–water in 30 min. Peak assignments as in Fig. 1. The line drawn across the chromatogram indicates baseline correction in the gradient of acetonitrile–water.

elution. The resolution (R_s) is very high for mannitol. Values of R_s higher than 2 permit one to identify clearly the different components present in the mixture.

This HPLC method was applied to lichen samples by using thalli of *E. prunastri* and *H. lugubris* floated on distilled water for 1 h in the dark. Fig. 4 shows a chromatographic sequence to determine whether in fact the peak which eluted between 8 and 9 min was ribitol or arabitol when extracts from *E. prunastri* were analysed. When the sample was loaded with 2.0 mg of ribitol (Fig. 4B), the absorbance at 195 nm of peak a did not increase. It is clear that this peak is arabitol because, as is shown in Fig. 4C, its absorbance increased on loading the sample with 2.0 mg of arabitol. Fig. 4D shows the result of applying the same procedure but loading the sample with 2.0 mg of mannitol. In this instance the absorbance of peak m increased 1.5-fold with respect to that without any added mannitol, showing that this peak is mannitol.

Chromatographic separation of the different components of *H. lugubris* extracts revealed similar polyols to those found in *E. prunastri*. A mannitol peak was evident whereas ribitol hardly appeared and arabitol was absent from these extracts (data not shown).

The composition of polyols extracted from both lichen species is shown in Table I. The procedure was highly reproducible, with standard errors lower than 5.0%. Here, and also in the chromatograms from *E. prunastri* extracts, peaks corresponding to fructose and glucose appeared as the only representative hexoses.

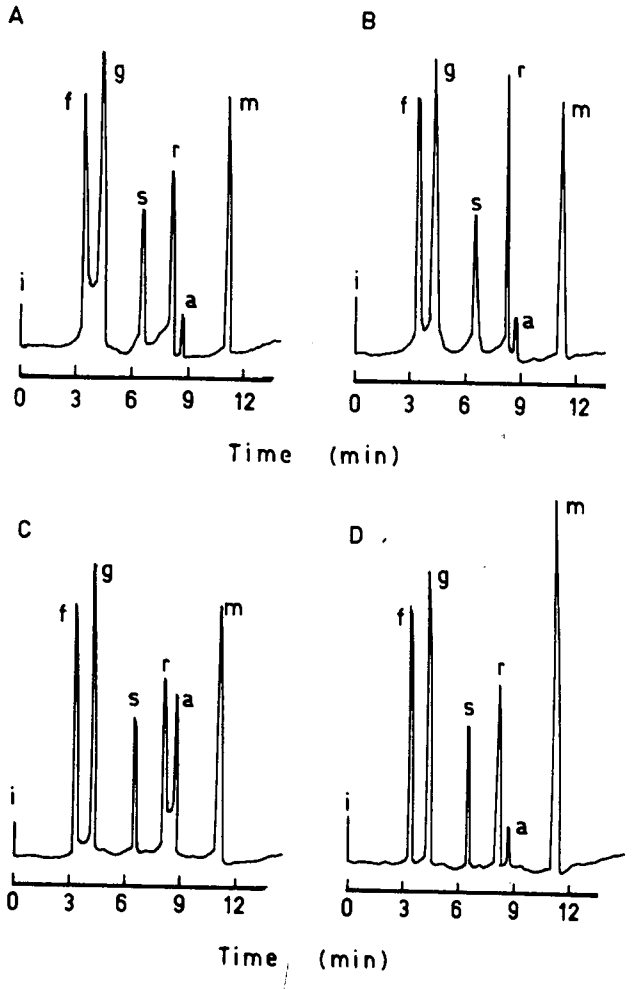

Fig. 4. (A) HPLC elution profile of an extract from the lichen *E. prunastri*. The column was eluted at 1.3 ml min^{-1} isocratically acetonitrile–water (80:20, v/v). Peaks: i = injection; f = fructose, g = glucose; s = D-ribose as internal standard; r = ribitol; a = arabitol; m = mannitol. (B), (C) and (D), similar profiles for extracts loaded with 2.0 mg of ribitol, arabitol and mannitol, respectively.

Separation of sugars

Unidentified peaks in both *Evernia* and *Himantormia* extracts were tested against chromatograms of several monosaccharides used as standards. By using the same procedure, and ribitol as internal standard, rhamnose, xylose, arabinose, fructose, mannose, glucose, galactose and sucrose were conveniently separated in less than 11 min (Fig. 5). The calibration lines, shown in Fig. 6 for four representative sugars, indicate that the method is linear over the range 1.0–100 μg injected.

Peaks with retention time of 3.2 and 4.3 min in the chromatographic trace of lichen extracts can be identified as fructose and glucose, respectively (Fig. 4A). On loading the lichen extracts with 2.0 mg ml^{-1} of fructose, only the peak with a reten-

TABLE I

QUANTITATIVE COMPOSITION OF POLYOL FRACTION ISOLATED FROM *EVERNIA PRUNASTRI* AND *HIMANTORMIA LUGUBRIS* THALLI AND ANALYSED BY HPLC

Polyol	Polyol content[a] (mg g^{-1} dry thallus)	
	Evernia prunastri	*Himantormia lugubris*
Ribitol	33.7 ± 1.2	1.3 ± 0.05
Arabitol	2.1 ± 0.08	n.d.[b]
Mannitol	1.6 ± 0.07	1.1 ± 0.04

[a] ± Standard error of four replicates.
[b] Not detected.

tion time of 3.24 min increases, whereas an increase in the second peak, with a retention time 4.3 min, only occurs when the sample is loaded with 2.0 mg ml^{-1} of glucose (data not shown).

It is to be expected that other plant extracts will show a more complex chromatographic behaviour on the basis of the well known contents of different sugars. The extract defined as the low-molecular-weight fraction obtained from sugarcane juices

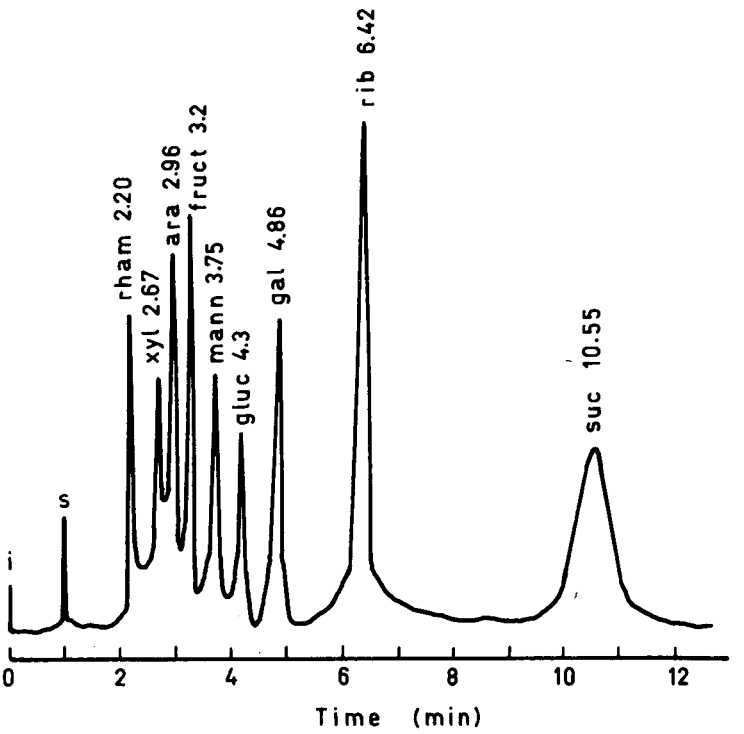

Fig. 5. HPLC separation of a standard mixture of D-rhamnose, D-xylose, D-arabinose, D-fructose, D-glucose, D-galactose, D-ribose and sucrose (2.0 mg of each in 1.0 ml of mobile phase). The column was eluted at 1.3 ml min^{-1} isocratically with acetonitrile–water (80:20, v/v).

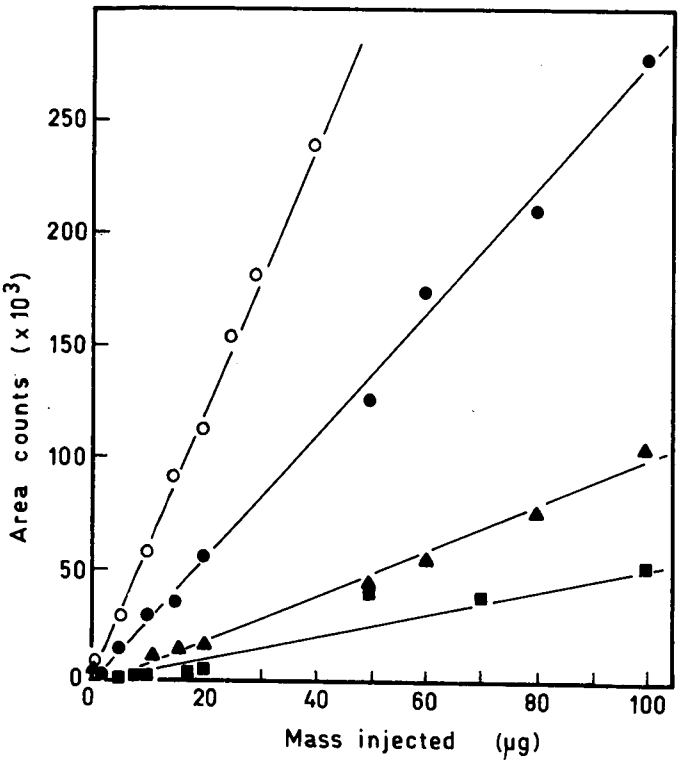

Fig. 6. Calibration lines for four representative monosaccharides from the standard mixture. Chromatographic conditions as in Fig. 1. Direct calibration of (○) ribose, $y = 5.9x - 4.88$, $r^2 = 0.99$, (●) fructose, $y = 2.73x - 3.12$, $r^2 = 0.99$, (▲) galactose, $y = 0.99x - 3.26$, $r^2 = 0.98$ and (■) glucose, $y = 0.56x - 3.66$, $r^2 = 0.98$. Data are the means of four replicates. The standard error was never larger than the symbols.

TABLE II

QUANTITATIVE COMPOSITION OF LOW-MOLECULAR-WEIGHT FRACTION ISOLATED FROM SUGARCANE JUICE BY FILTERING IT THROUGH SEPHADEX G-10 AND ANALYSED BY HPLC

Sugar	Concentration (μg ml^{-1})	Percentage of the fraction	Percentage of the total juice
Xylose	4.87 ± 0.46	7.23	1.95
Arabinose	6.12 ± 0.54	9.08	2.45
Galactose	4.12 ± 0.39	6.11	1.65
Fructose	3.18 ± 0.42	4.71	1.27
Glucose	2.66 ± 0.19	3.95	1.06
Sucrose	18.51 ± 1.64	27.50	7.43
Totals	39.46	58.58	15.81

[a] ± Standard error of four replicates.

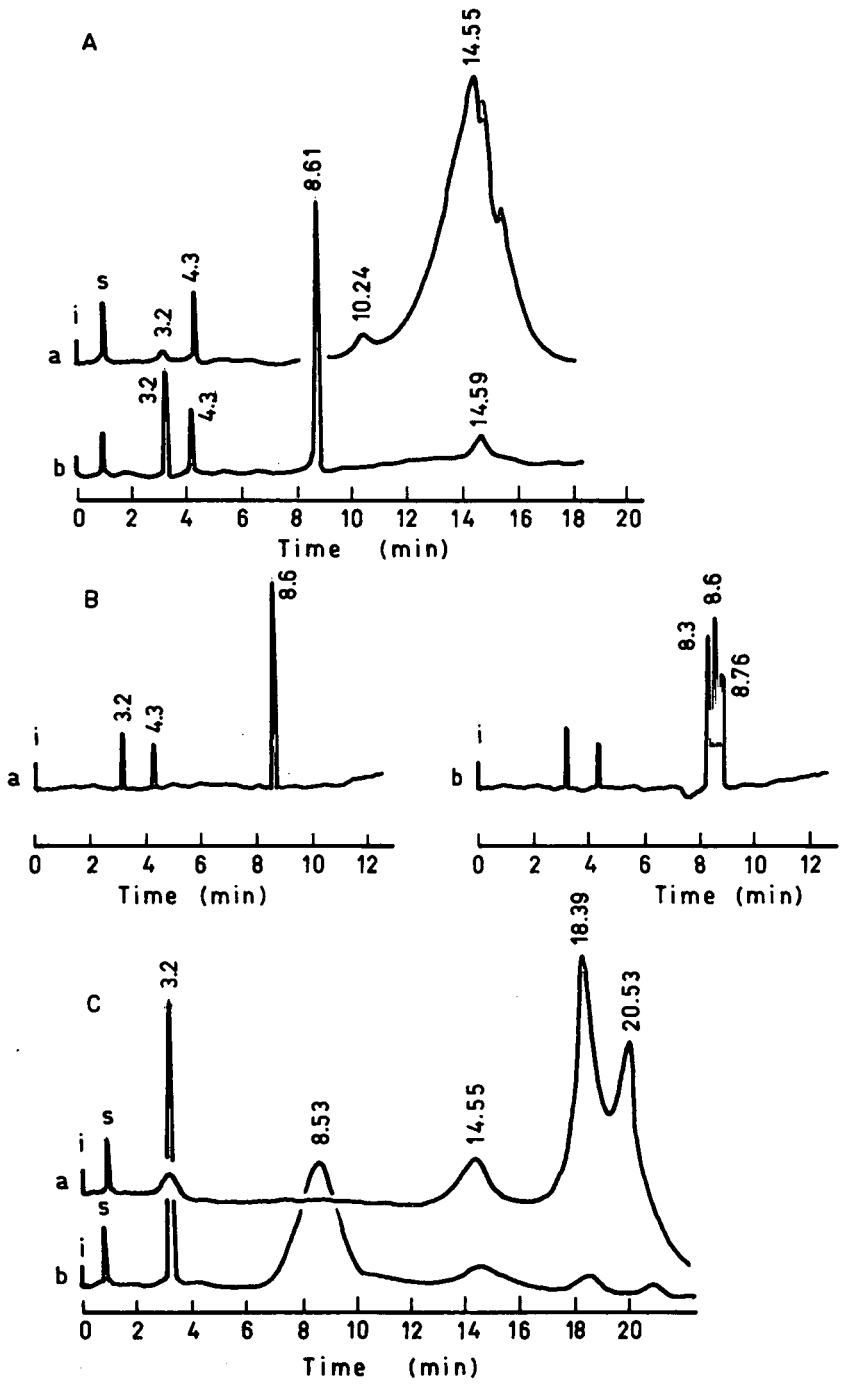

Fig. 7. (A) HPLC elution profiles of MMWC fraction isolated by Sephadex G-10 and G-50 filtration of clarified sugarcane juice (a) before and (b) after acidic hydrolysis. Peaks: i = injection; s = solvent. Numbers on the peak are retention times (min). (B) HPLC elution profiles of a hydrolysate of MMWC loaded with (a) 2.0 mg ml^{-1} of galactitol or (b) 2.0 mg ml^{-1} of ribitol or arabitol. (C) HPLC elution profiles of SP fraction isolated by Sephadex G-10 and G-50 filtration of clarified sugarcane juice (a) before and (b) after acidic hydrolysis.

as described under Experimental gave peaks with retention times of 2.20, 2.67, 3.2, 4.3, 4.86 and 10.55 min. On loading the samples with 2.0 mg ml^{-1} of the different standards, fructose [retention time (t_R) = 3.2 min], glucose (t_R = 4.3 min) and sucrose (t_R = 10.55 min) were identified. Xylose (t_R = 2.2 min), arabinose (t_R = 2.63 min) and galactose (t_R = 4.91 min) were identified by using the same procedure. Although sucrose represent about 27.5% (dry weight) in this fraction, xylose, arabinose and galactose are the main monosaccharide forms (Table II).

Nature of sugarcane juice polysaccharides

The filtrate from the Sephadex G-50 column defined as the MMWC fraction contains traces of glucose, fructose and sucrose [not more than 0.67% (dry weight) of each] and a large peak with a retention time of 14.55 min (Fig. 7A, trace a). This peak possibly represents the major polysaccharide form in this fraction. In contrast, the filtrate defined as SP seemed to be composed of a heterogeneous pool of polymeric forms, with retention times of about 14.55, 18.4 and 20.63 min (Fig. 7C, trace a). Both the SP and MMWC fractions were then dried in air and hydrolysed with 6 *M* hydrochloric acid for 2 h at 60°C. The hydrolysates were dried in air to remove the acid and extracted as described above. The residues were dissolved in a sufficient volume of acetonitrile–water (80:20, v/v) to give a final concentration of 1.0 mg ml^{-1} dry residue and analysed by HPLC.

The peak with a retention time of 14.55 min disappeared after acidic hydrolysis of MMWC and, simultaneously, the amount of fructose increased and a new peak appeared with a retention time of *ca.* 8.6 min (Fig. 7B, trace a), which was tentatively identified as galactitol. By loading the sample with 2.0 mg ml^{-1} of different polyols and sugars, this peak only increased when galactitol was added (Fig. 7B, trace b). The SP fraction, after acidic hydrolysis, was found to be composed only of galactitol and fructose (Fig. 7C, trace b).

DISCUSSION

Many techniques have been described previously for sugar and polyol analysis. However, HPLC allows extremely accurate detection and determination. The decision about the column to be used is complex and requires the evaluation of various factors. In general, non-destructive detection (UV), inexpensive solvents, short retention times and high sensitivity of the detector can be advantageous.

Over the last decade, a number of HPLC methods for separating reducing sugars have been reported, although there are few methods for separating polyols [18–20]. Even using HPLC procedures, MacFarlane and Kershaw [11] emphasized the difficulty in the separation of mannitol and arabitol, perhaps because the refractive index detector used did not permit gradient elution. In previous papers [17,21], we reported the separation of ribitol, arabitol and mannitol as trimethylsilyl derivatives by GLC, although ribitol and arabitol were eluted as a single peak.

We have reported here the complete separation of ribitol from mannitol and arabitol with R_s values of *ca.* 4 under isocratic conditions, and the use of a linear gradient decreased the R_s values by 1 unit. In addition, the use of a UV detector substantially improved the determination and the linearity of the detector response. A convenient separation in the isocratic mode was also achieved for sugars. Isocratic

separation of mono- and oligosaccharides by HPLC have also been reported previously for plant extracts [22], medical formulations [23] and several hexosamines [24]. Fructose and glucose, identified by this procedure in both *E. prunastri* and *H. lugubris* extracts, are the main components of the monosaccharide fraction of many lichen species [11].

Arabinose, xylose and galactose have been detected in sugarcane juice as normal constituents of low-molecular-weight sugars separated by filtration of clarified juice through a Sephadex G-10 column (Table II). Their concentrations are higher than those of both glucose and fructose but, obviously, lower than that of sucrose. Glucose and fructose have been described as normal components of fresh crushed juice [5,12] and raw sugar [25]. In the latter, xylose also appears to be a constant constituent. However, there is no report of the occurrence of both arabinose and galactose in crude juice as free monosaccharides, although they are the main products of hydrolysis (analysed by GLC) of high-molecular-weight polysaccharides, defined as arabinogalactans [26]. Hence it is possible that both monosaccharides were produced by hydrolysis during the processing of sugarcane stalks or juice. In spite of this, other polysaccharides have been detected without any significant hydrolysis. Therefore, it is unlikely that a single polysaccharide can be completely hydrolysed during juice storage whereas others are not. In addition, separation of monosaccharides by HPLC from sugarcane juices, reported previously, did not indicate components other than glucose and fructose because only water was used as the mobile phase at a high flow-rate (higher than 2.0 ml min^{-1}) to ensure a good resolution of sucrose [5]. Under these conditions, many peaks eluting before 6 min can be superimposed on that of glucose and fructose. Probably the use of acetonitrile as a component of the mobile phase improves the separation of sugars by increasing their retention.

The fractions defined here as MMWC contain a main polysaccharide which is composed of fructose and galactitol, as was revealed by acidic hydrolysis. A small amount of sucrose contaminates these fractions eluted from Sephadex G-50, and it is responsible for the formation of glucose after hydrolysis. However, the amount of fructose that appears in the hydrolysate cannot be derived only from sucrose. Hence a large amount of this fructose must be produced from polysaccharide hydrolysis. In any case, the quantitative decrease of the peak with a retention time of 14.55 min confirms that this hydrolysis has been achieved.

An unexpected result of this hydrolysis was the production of a compound that has been identified as a polyol. Production of polyols, such as mannitol, has previously been reported from deteriorated sugarcane juices [5], but the simultaneous production of both ethanol and lactic acid clearly indicates that the juices contain products of a fermentation process. These juices were completely different from those, freshly produced, used in this work. However, the peak of galactitol was perfectly identified as it was the only peak that increased after loading the sample with exogenous galactitol and, in addition, the identity of the products of hydrolysis of both MMWC and SP are very significant. It is possible that the resistance to enzymatic hydrolysis of a fraction of juice polysaccharides when bacterial dextranases or glucanases are used [27] can be achieved by including a polyol in the polymeric sequence.

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Evaluation of different packings for high-performance liquid chromatographic analysis of alkyl lysophospholipids

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ABSTRACT

The analysis of the alkyl lysophospholipid 1-octadecyl-2-O-methyl-D,L-glycero-3-phosphorylcholine is currently under investigation because of its anticancer activity. The chromatographic behaviour of this compound and its 1-hexadecyl-2-O-methyl-D,L-glycero-3-phosphorylcholine homologue, which is used as an internal standard for pharmacokinetic studies, on various liquid chromatography packings gave rise to many problems. The retention and elution characteristics of both ether phospholipids were studied on silica, straight polyethyleneglycol-coated silica, reversed-phase materials, base-deactivated reversed-phase silica and polymeric resins.

INTRODUCTION

1-Octadecyl-2-O-methyl-D,L-glycero-3-phosphorylcholine (Et-18-OMe) belongs to a family of new antitumour drugs. The group of alkyl lysophospholipids (ALPs) contains synthetic analogues of the naturally occuring 2-lysophosphatidylcholine which are selectively cytotoxic for tumour cells [1,2]. To study the influence of Et-18-OMe concentration and the effect of its metabolism in cells on antitumour activity, precise analytical methods are required. The 1-hexadecyl-2-O-methyl-D,L-glycero-3-phosphorylcholine (Et-16-OMe) homologue is an inactive derivative, which can be used as an internal standard.

Capillary gas chromatography has been investigated for the analysis of ether phospholipids. The technique appears to be very attractive because of its intrinsically high resolving power and high sensitivity. However, dephosphorylation is required and this reaction is far from being quantitative [3]. High-performance thin-layer chro-

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matography with fluorescence detection is a fast and simple analytical technique for this class of compounds, but lacks sensitivity [4].

High-performance liquid chromatography (HPLC) is a well known technique for the differentiation of lipid and phospholipid classes. The HPLC of phospholipids is usually performed by straight-phase liquid chromatography [5–8]. The separation is based on polarity differences between the phospholipid classes and not on chain length. However, the use of normal-phase silica is associated with problems of column stability and the reproducibility of retention data. The work reported here evaluated different reversed-phase materials, and the problems encountered and the selectivity characteristics of the columns for the separation of Et-16-OMe and Et-18-OMe are described in this paper.

EXPERIMENTAL

A Perkin-Elmer Series 4 liquid chromatograph, equipped with a Perkin-Elmer LC terminal and a Series 4 control module, was used in combination with a D.D.L. 11 light scattering detector (Sedere, France). Nebulization was performed using air at 2 bar and 40°C. Peak registration was performed with a Varian A-25 recorder or a Shimadzu C-R6A Chromatopac integrator. For refractive index determination an R401 differential refractometer (Waters Assoc., U.S.A.) coupled to a Waters M-45 pump was used. The samples were injected using a Valco valve with a 25-μl loop.

Columns

Normal-phase LC was performed on a LiChroma column (A), 150 × 4.6 mm, 5 μm (Alltech, Belgium) and on polyethyleneglycol (PEG)-coated silica (B), 150 × 4.6 mm, 5 μm. The method using the PEG coating was performed according to the procedure of Ghijs *et al.*[9].

LiChrosorb RP-2 (C) (150 × 4.6 mm, 5 μm) was supplied by Alltech; LiChrosorb RP-8 (D) and RP-18 (E) (150 × 4.6 mm, 5 μm) and Polygosil-60-D-10-CN (F) (250 × 4.6 mm, 10 μm) were purchased from Chrompack (Belgium). LiChrosorb 60 RP Select B (G) (Merck, Germany) contains base-deactivated silica with a pore size of 60 Å instead of 100 Å (150 × 4.6 mm, 5 μm). The phase was not end-capped, but specially treated to obtain the maximum bonding of methyloctyl groups [10]. An Apex Symm columm (H) (150 × 4.6 mm, 5 μm) was used which contained silica-based octadecyl reversed-phase material, which is claimed to be base-deactivated (Jones Chromatography, U.S.A.). Ultrabase C_{18} (I) (250 × 4.6 mm, 5 μm) (Chromatech, France) has been developed for the same reason. Vydac 201 TP 54 (J) (250 × 4.6 mm, 10 μm) (Separations Group, Hesperia, CA, U.S.A.) is a polymeric octadecyl phase.

PRP-1 (K) is a macroporous styrene divinylbenzene copolymer (Hamilton, Switzerland). The column dimensions are 150 × 4.1 mm, 5 μm. PRLP-S (L) 100 Å (250 × 4.6 mm, 5 μ,) is a rigid polystyrenedivinylbenzene without a hydrophobic ligand, and was supplied by Polymer Labs. (Separation Science Division, U.K.).

Reagents

Et-18-OMe (clinical grade) was obtained from Medmark Pharma (Germany). Et-16-OMe and Et-18-OMe (Fig. 1) (analytical grade) were supplied by Sigma

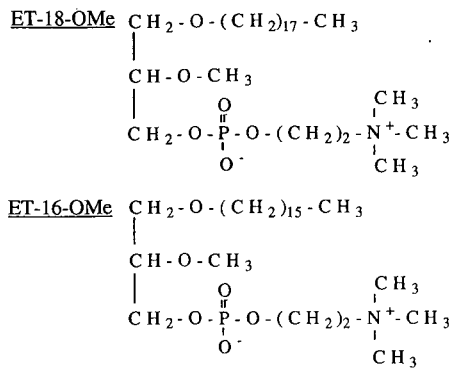

Fig. 1. Chemical structures of Et-16-OMe and Et-18-OMe.

(U.S.A.). The mobile phases were of HPLC grade. Other products and solvents used were of analytical-reagent grade and were used without further purification. Deionized, distilled water was used throughout.

RESULTS AND DISCUSSION

Adsorption chromatography

Silica has been used for many years as a stationary phase in liquid–solid chromatography. The retention of the solutes is mainly based on hydrogen bonding, dipole–dipole interactions and ion-exchange mechanisms. The majority of silanol functions are weakly acidic, although a small portion is strongly acidic and is even ionized at low pH. A weak acid silica phase (pK_a = 7.0) thus exhibits properties typical of weakly acidic cation exchangers.

Using the mobile phase chloroform–methanol–water (60:34:5,5, v/v), no elution of the ALPs was observed. However, the addition of 0.5% (v/v) strong ammonia allowed the elution of the adsorbed ether phospholipids. The ammonia forms a protective shield for the active silanols.

The ALPs have a completely different physico-chemical behaviour from the more hydrophobic natural phospholipids such as lecithin. Mobile phase based on hexane, chloroform and methanol mixtures, which are very popular for the chromatography of these compounds, are too apolar to elute the ALPs. A high water content in the eluent is necessary to obtain elution in a reasonable time.

The separation of the two structurally related compounds, Et-16-OMe and Et-18-OMe on silica gel cannot be achieved as the ion-exchange mechanism is similar for both compounds. It has been suggested, however, that the silica surface has different kinds of silanol groups, *e.g.*, free (isolated) or hydrogen-bonded, each with different physico-chemical activities which affect the chromatographic behaviour differently. Siloxane bridges are frequently assumed to be inactive or to contribute to retention by a hydrophobic chromatographic mechanism. The difference in hydrophobicity of the two homologues is not substantial enough to obtain a better separation than that shown in Fig. 2. Decreasing the polarity by diminishing the water content does not improve selectivity, but only results in band broadening.

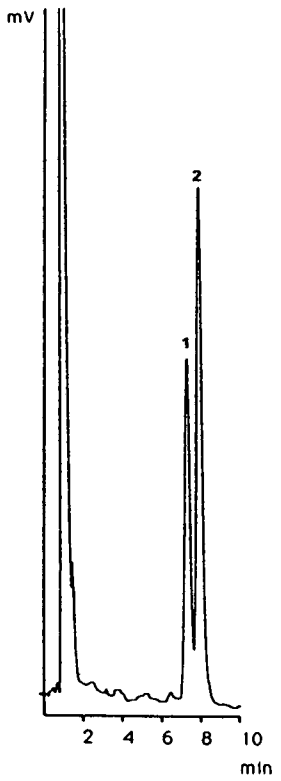

Fig. 2. Separation of (1)Et-18-OMe and (2)Et-16-OMe on column A (LiChroma silica). Conditions: mobile phase, chloroform–methanol–water–ammonia (60:34:5.5:0.5, v/v); flow-rate, 1.0 ml/min; sample loop, 20 μl; and concentration mixture, *ca.* 250 μg/ml.

Under the same chromatographic conditions, the resolution of these two compounds was not constant. Fig. 2 shows the best separation obtained. As silica is characterized as a "natural polymer" it is not unreasonable to assume that the silanols and siloxane groups exist in some kind of slow equilibrium. Therefore it is not surprising that the chromatography performed on silica-based materials is not always reproducible. It is also well known that the use of silica gel is always correlated with problems in column stability.

Coating silica with PEG changes the properties of the phase such that ALPs can no longer be adsorbed and are eluted without ammonia. However, no substantial influence on selectivity was observed as the main mechanism of separation is still based on polarity difference between both compounds (Fig. 3).

Reversed-phase chromatography

Alkyl-bonded silica is the material of choice for a large number of chromatographic separations. Silica is mechanically rigid, which makes it well suited for reversed-phase chromatography where a small particle size and high pressures are required. Many of the benefits of reversed-phase LC are derived from the properties

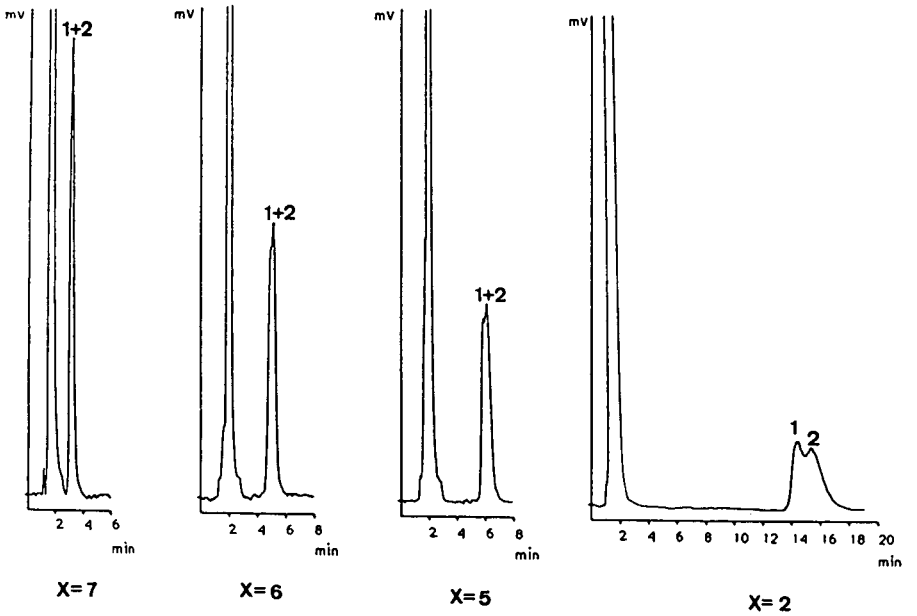

Fig. 3. Separation of (1) Et-18-OMe and (2) Et-16-OMe on column B (PEG). Conditions: mobile phase, chloroform–methanol–water (60:34:*x*, v/v); flow-rate, 1.0 ml/min; sample loop, 20 μl; and concentration mixture, *ca.* 200 μg/ml.

TABLE I

COMPARISON OF RETENTION CHARACTERISTICS OF ET-16-OME (16) AND ET-18-OME (18).

Columns used were C to L with mobile phases of methanol and methanol containing 0.5% (v/v) strong ammonia solution. Number of experiments = 3.

Column	Methanol			Ammonia solution–methanol (0.5:99.5, v/v)		
	k'_{16} [a]	k'_{18} [b]	α [c]	k'_{16} [a]	k'_{18} [b]	α [c]
C	∞	∞	–	4.356	4.356	1.000
D	∞	∞	–	1.258	1.348	1.072
E	∞	∞	–	1.528	2.067	1.353
F	0.961	0.961	1.000	1.046	1.046	1.000
G	1.103	1.224	1.110	1.103	1.224	1.110
H	∞	∞	–	∞	∞	–
I	2.607	3.607	1.384	2.643	3.786	1.432
J	∞	∞	–	∞	∞	–
K	1.371	1.924	1.404	1.289	1.871	1.452
L	1.377	1.926	1.399	1.372	1.936	1.411

[a] Capacity factor for Et-16-OMe.
[b] Capacity factor for Et-18-OMe.
[c] Selectivity, as defined in text.

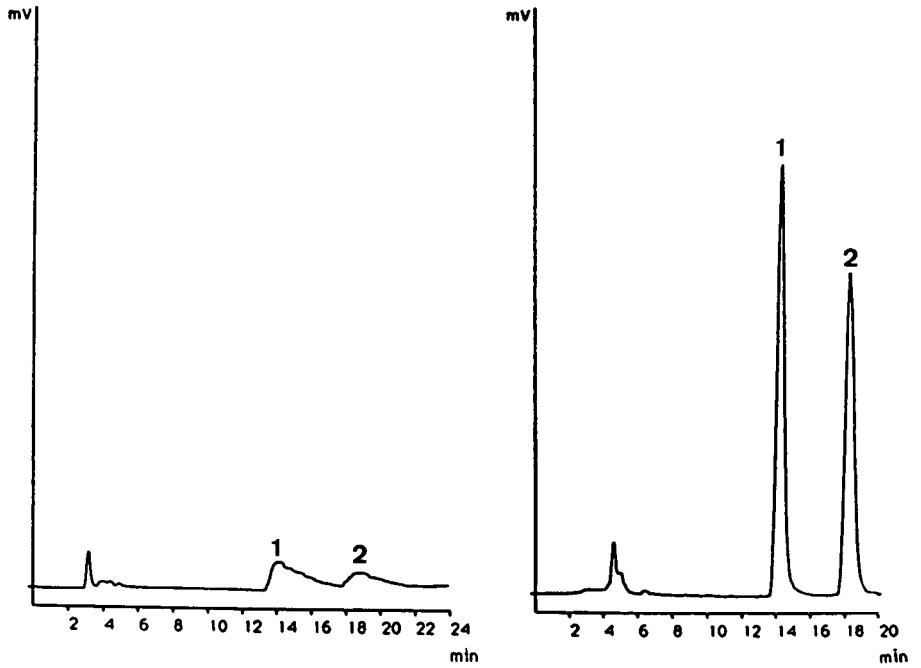

Fig. 4. Separation of (1) Et-16-OMe and (2) Et-18-OMe on column H (left) (Apex Symm RP18) and I (right) (Ultrabase C18). Conditions: mobile phase, 100% methanol; flow-rate 1.5 ml/min; sample loop, 20 μl; and concentration mixture, *ca.* 250 μg/ml.

of silica. The separation mechanism is attributed to hydrophobic interactions between the solutes and the alkyl chains of the chemically modified silica gel.

Isocratic elution with methanol on RP-2 (C), RP-8 (D) and RP-18 (E) phases was insufficient for the separation of the two studied compounds. This can be attributed to interactions with free silanol groups. These silanols are highly acidic and are very reactive for the quaternary ammonium group. The adsorption or desorption of the phosphocholine moiety by the silanols has slow reaction kinetics. The effect of such silanophilic interactions on retention has been tested for other recently introduced HPLC materials. Manufacturers claim that their materials posses the benefits of silica-based reversed phases but with a minimized silanol activity.

The various retention characteristics are summarized in Table I, in which capacity factors and selectivities are given. Capacity factors (k') are measured by the relationship $(t_R - t_0)/t_0$, where t_R is the retention time of the compound of interest and t_0 is the column void volume measured using the solvent disturbance peak when methanol is injected onto the column. Selectivities (α) for the studied ALPs are calculated by the formula k'_2/k'_1, where k'_2 is the capacity factor of the compound with the highest retention and k'_1 is the capacity factor of the compound with the lowest retention.

It should be noted that the Apex Symm column (H), in contrast to the Ultrabase C 18 analogue (I), shows a low efficiency (Fig. 4). To obtain a good selectivity, an octadecyl coating is necessary. The hydrophobic affinity between the octyl chains and

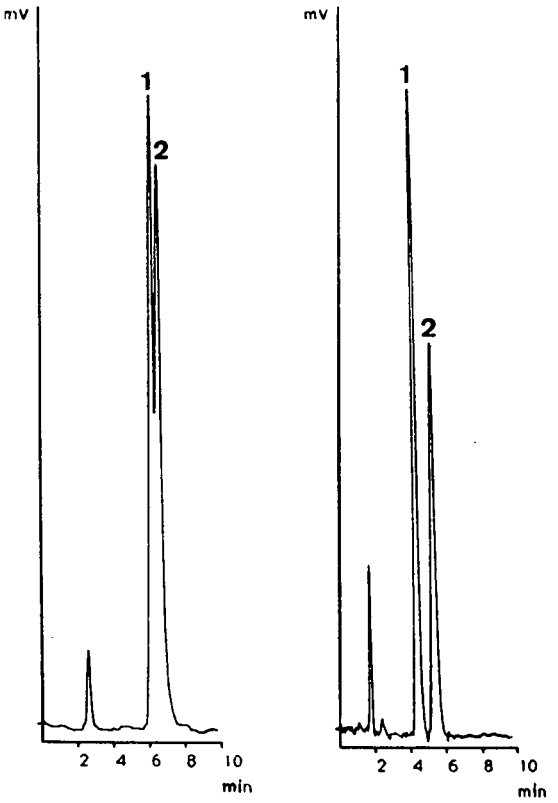

Fig. 5. Separation of (1) Et-16-OMe and (2) Et-18-OMe on column G (left) (LiChrosorb 60 RP Select B) and E (right) (LiChrosorb RP-18). Conditions: mobile phase 100% methanol (column G), strong ammonia solution–methanol (0.5:99.5, v/v) (column E); flow-rate 1.0 ml/min; sample loop, 20 μl; and concentration mixture *ca.* 200 μg/ml.

the long alkyl chain of the ALPs is too low to provide sufficient separation (Fig. 5).

Current methods to reduce the base–silanol interaction not only consist in modifying the stationary phase, but also in mobile phase modification. This includes pH changes or increasing the ionic strength of the mobile phase to suppress ionization and ionic interactions and the addition of low-molecular-weight amines to compete with the analyte base for adsorption on to the acidic silanols. In fact, good chromatographic practice often requires masking the surface silanols by doping the eluent with low concentrations of an aliphatic amine, such as triethylamine (TEA) to reduce peak tailing. The use of such 'modifiers' is restricted because there are limitations on the range of materials which can be added to the mobile phase when light scattering is used as a detection method. For the same reason, ion-pair reagents such as butanesulphonic acid could not be used in the ion-pair chromatographic separation of these quaternary ammonium compounds. Only ammonia and acetic acid could be added to the eluent without the mass detector moving off-scale. Doping methanol with 0.5% acetic acid (v/v) to suppress the ionization of the silanols was not succesful.

Ammonia obviously masked the silanols in the same way as it did in the

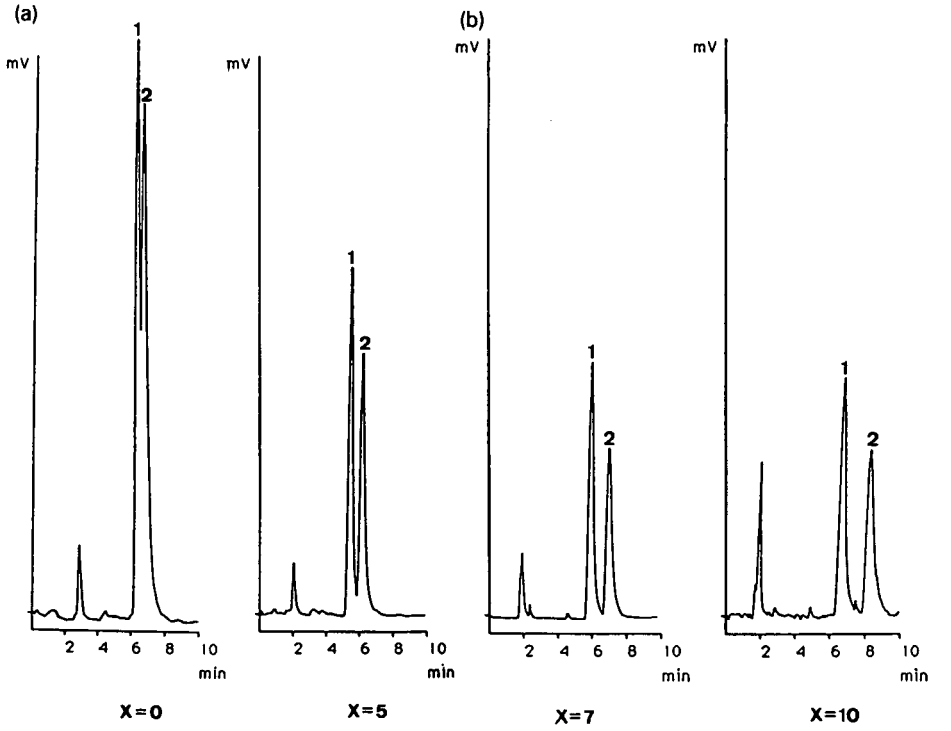

Fig. 6. Separation of (1) Et-16-OMe and (2) Et-18-OMe on Column D (Lichrosorb RP-8). Conditions: mobile phase, strong ammonia solution–water–methanol (0.5:variable, v/v); flow-rate, 1.5 ml/min; sample loop, 20 μl; and concentration mixture, *ca.* 200 μg/ml. x = % water (v/v).

straight-phase mode. The results are shown in Table I. Most of the reversed phase columns, which showed adsorption without ammonia, now eluted with ammonia. The adsorption problem on the Vydac (I) and base-deactivated Apex Symm column (H) was not solved. This proves again that the optimization and standardization of silica packing materials in LC is difficult. The possible correlation between ammonia concentration and the reversibility of adsorption of the ALPs was investigated. Improvements in elution and selectivity were observed when the RP-8 column (D) was gradually "loaded" by increasing the amount of ammonia. However, it was difficult to see such a dependence because the elution is obtained only after loading the column with an excess of ammonia.

The ethyl (C), octyl (D, G) and cyanopropyl (F) phases are not sufficiently hydrophobic to create an affinity for the long ether chain; the selectivities are summarized in Table I. The resolution on the octyl reversed-phase column could be improved by adding water to the eluent. Fig. 6 shows four chromatograms obtained after elution on a classical RP-8 column (D) with increasing amounts of water. The effect of increasing the polarity on the retention and separation on RP-8 (D) and base-deactivated RP-8 (G) is summarized in Table II.

When methanol was replaced by acetonitrile on the RP-8 and RP-18 phases, the good elution profiles were no longer observed. This is not due to a detection problem

TABLE II

INFLUENCE OF WATER CONTENT IN THE MOBILE PHASE ON THE RETENTION MECHANISMS OF ET-16-OME (16) AND ET-18-OME (18) ON RP-8 (D) AND LICHROSORB 60RP SELECT B (G) COLUMNS

Number of experiments = 3. Symbols as in Table I.

Water (%, v/v)	Column D			Column G		
	k'_{16}	k'_{18}	α	k'_{16}	k'_{18}	α
0	1.040	1.161	1.116	1.103	1.224	1.110
5	1.698	2.073	1.221	1.810	2.293	1.267
7	1.929	2.449	1.269	2.478	3.400	1.372
10	2.544	3.422	1.345	3.690	5.466	1.481

because the same phenomenon was observed when refractive index detection was used instead of light scattering. This observation can be explained by the fact that ALPs are not very soluble in acetonitrile–water.

Another chromatographic approach could be the use of polymeric particles such as polystyrenedivinylbenzene. No data for the chromatography of phospholipids with these materials have yet been reported. The chromatographic separation of Et-16-OMe and Et-18-OMe was therefore tried on two commercial phases, PRP-1 (K) and PRLP-S (L). The columns seem to perform very well for this type of separation because no adsorption was observed (see Table I) and the chromatographic efficiency was better than with octyl reversed phases. The microporosity of this resin explains the greater hydrophobic properties of polystyrenedivinylbenzene compared with the classical octyl or octadecyl reversed phases. The influence of the addition of water which causes a dramatic increase in the retention of the ALPs because of the reduced hydrophobicity in the microporous cavities, is also seen.

However, when water was added to methanol, a strange phenomenon occurred. Although there is no obvious explanation for this behaviour, it is reported here. The results are summarized in Table III and chromatograms are shown in Fig. 7. When

TABLE III

INFLUENCE OF WATER CONTENT IN THE MOBILE PHASE ON THE RETENTION MECHANISMS OF ET-16-OME (16) AND ET-18-OME (18) ON A PRLP-S- COLUMN (L)

Number of experiments = 3. Symbols as in Table I.

Water (%, v/v)	k'_{16}	k'_{18}	α
0	1.373	1.926	1.399
1	1.500	2.182	1.455
2	1.831	2.712	1.481
3	2.049	3.223	1.573
4	2.466	4.095	1.661
5	2.769	4.758	1.718
6	2.826	4.871	1.724
8	4.776	9.265	1.940
10	6.197	12.826	2.070

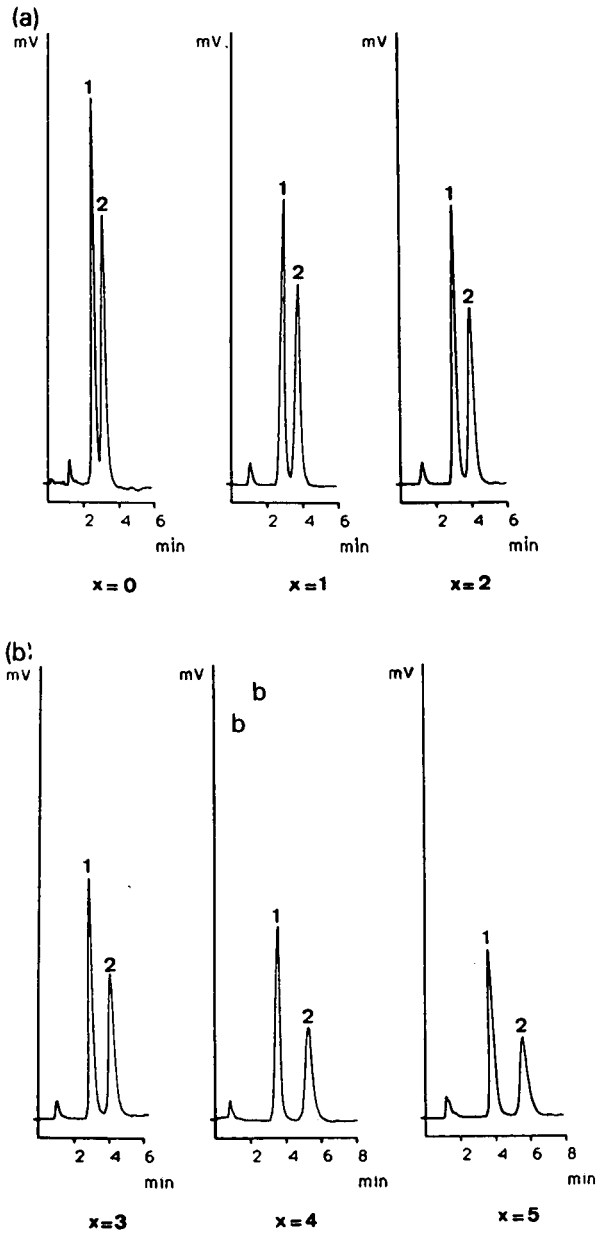
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Fig. 7.

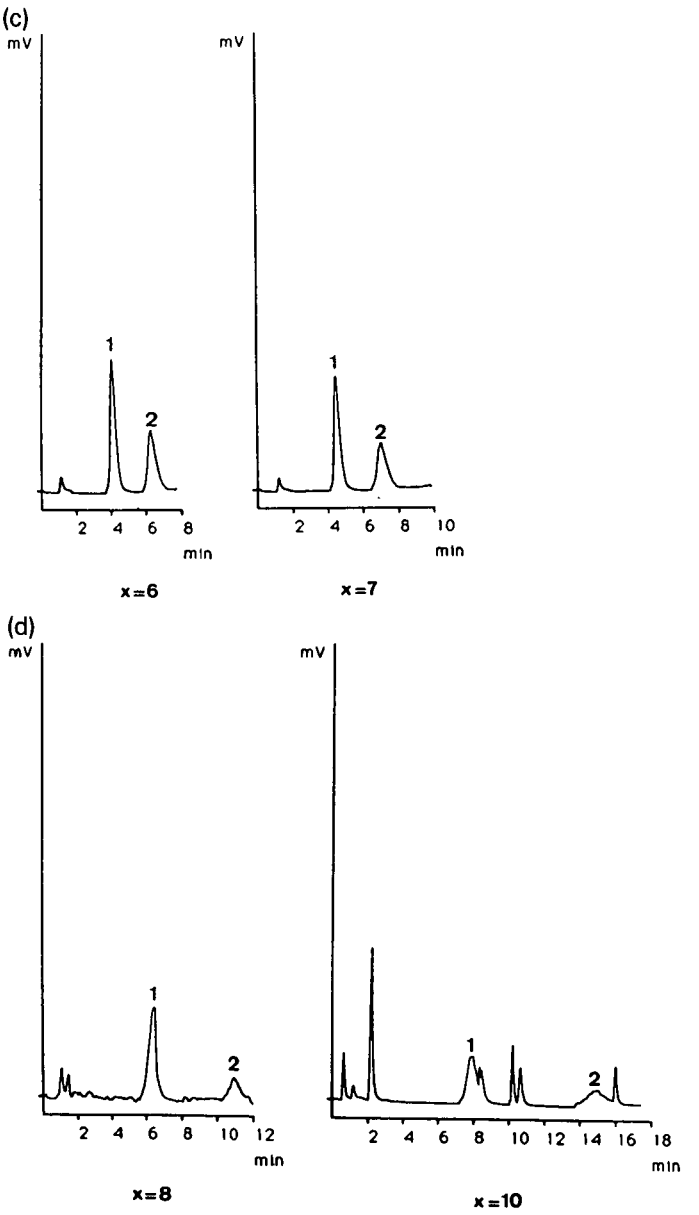

Fig. 7. Separation of (1) Et-16-OMe and (2) Et-18-OMe on Column L (PRLP-S). Conditions: mobile phase, water–methanol (variable); flow-rate, 1.0 ml/min; sample loop, 20 μl; and concentration mixture, *ca.* 200 μg/ml. x = % water (v/v).

the water content is increased, the resolution is improved but the observed eluted amount decreases. The last chromatographic run with 10% (v/v) water in methanol yielded a peak distortion. Fig. 8 shows the dependence of the addition of water to the mobile phase on the detection signal. The peak height and surface area decreased

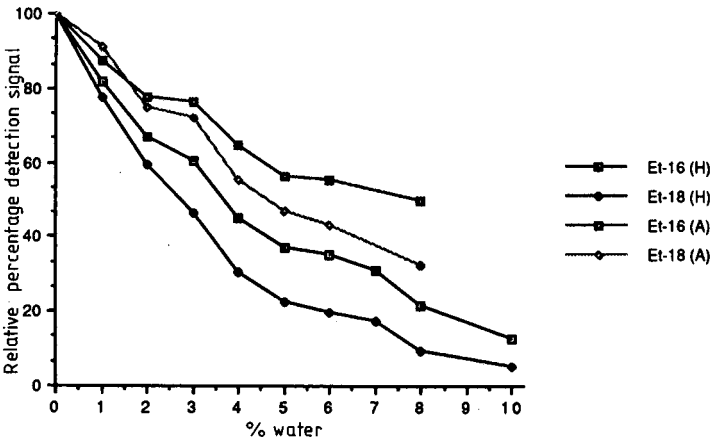

Fig. 8. Relative percentage detection signal compared to the values obtained in the chromatogram on column L (PRLP-S) with 0% water. (H) peak height; (A) area under the curve.

significantly as a function of water content. Chromatography on polystyrenedivinylbenzene is impossible with the mobile phase methanol–water (100:10, v/v) whereas it is still possible on reversed-phase materials (Fig. 6).

CONCLUSIONS

The best results for the separation of the two ALPs studied were obtained on the Ultrabase column (I), where the selectivity was highest and where no silanol activity was observed. The proposed HPLC method with light scattering detection can be used for studies of relatively high-concentration ALP formulations. However, as it is intended to develop an analytical method for the determination of ALPs in pharmacokinetic studies, where sensitivity is of prime importance, the possibilities of capillary gas chromatography are currently being investigated.

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CHROMSYMP. 2130

Rapid determination of abamectin in lettuce and cucumber by high-performance liquid chromatography

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ABSTRACT

A rapid, sensitive and reliable method is presented for the determination of trace amounts of abamectin in lettuce and cucumber. Abamectin consists of $\geqslant 80\%$ avermectin B1a and $\leqslant 20\%$ avermectin B1b. Vegetables were extracted with ethyl acetate and the extract was purified by solid-phase extraction using Sep-Pak silica cartridges. The purified extracts were analysed by high-performance liquid chromatography with a 5-μm Zorbax ODS column and UV detection under isocratic conditions. The method yields recoveries for avermectin B1a and B1b of 76–109% in the 0.054–0.54 mg/kg range. The limit of detection of the method is 40 μg/kg each of avermectin B1a and B1b in vegetables.

INTRODUCTION

The avermectins, a family of pesticidal agents, are macrocyclic lactones produced by the actinomycete *Streptomyces avermitilis* [1]. The avermectin structures were elucidated by Albers-Schönberg *et al.* [2], and some of the biological activities have been reviewed [3]. Abamectin is the commercial product that is being developed as an insecticide and acaricide with contact and stomach action. Abamectin consists of $\geqslant 80\%$ avermectin Bla and $\leqslant 20\%$ avermectin Blb (Fig. 1).

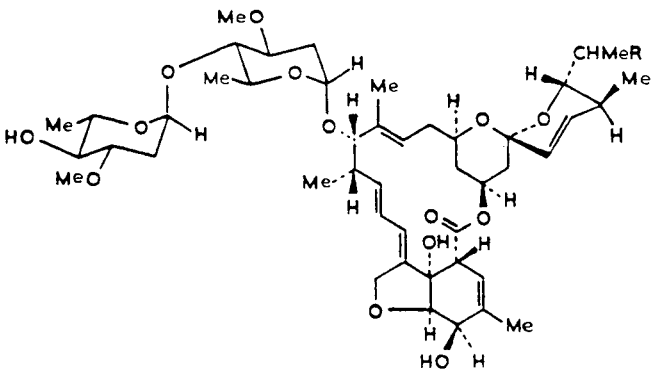

Fig. 1. Structures of the two major components of abamectin: Bla ($R = C_2H_5$), $\geqslant 80\%$, and Blb ($R = CH_3$), $\leqslant 20\%$. Me = CH_3.

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Several methods have been described for determining avermectins. Tolan *et al.* [4] and Tway *et al.* [5] described the determination of avermectins in plasma and ivermectin in tissue, respectively. Their methods are sensitive but also time consuming because of the elaborate clean-up steps and derivatization required. Fox and Fink [6] described the determination of ivermectin in feeds by high-performance liquid chromatography (HPLC). However, the method is not sensitive (detection limit 6 mg/kg). Iwata *et al.* [7] and Maynard *et al.* [8] described the determination of avermectin Bla in citrus fruits. The methods are sensitive (detection limits 0.1 and 5 μg/kg, respectively), but radiolabelled avermectin (^{3}H and ^{14}C, respectively) were used.

The purpose of this study was to develop a rapid, sensitive and quantitative method for the determination of abamectin (avermectin Bla and Blb) in lettuce and cucumber. The vegetables were extracted with ethyl acetate and the extracts were purified by solid-phase extraction. Separation and detection were performed using reversed-phase HPLC with ultraviolet detection. Recovery experiments were carried out in lettuce and cucumber.

EXPERIMENTAL

Reagents

All reagents were of analytical-reagent grade. Ethyl acetate, hexane and methanol were obtained from Merck (Darmstadt, Germany). The solid-phase extraction was done with Sep-Pak silica cartridges (0.8 g) obtained from Waters Assoc. (Milford, MA, U.S.A.). An abamectin standard in glycerol containing 1.074% (w/w) avermectin Bla and 0.125% (w/w) avermectin Blb was obtained from Merck Sharp & Dohme (Haarlem, The Netherlands). Abamectin standard solutions were prepared by diluting a stock standard solution of abamectin containing 54.0 mg/l Bla and 6.28 mg/l Blb in methanol. The standard solutions were stored in the dark at 4°C. Under these conditions the standard solutions were stable for at least 4 months.

Extraction procedure

The vegetables were chopped with a cutting machine (Stephan). A 50-g amount of chopped vegetables was weighed into a 250-ml centrifuge tube and 100 ml of ethyl acetate were added. The mixture was macerated with an Ultra Turrax for 1 min. The extract was centrifuged at 2000 *g* for 1 min and 50 ml of the supernatant were transferred to a round-bottomed flask and evaporated to dryness on a rotary evaporator (Buchi 011) at 40°C. The residue was dissolved in 2 ml of ethyl acetate, then 3 ml of hexane were added. The contents of the flask were mixed and applied to a Sep-Pak silica cartridge. The flask was washed twice with 1 ml of 40% ethyl acetate in hexane and the washings were applied to the cartridge. The cartridge was first washed with 8 ml of ethyl acetate–hexane (40:60), then was eluted with 5 ml of ethyl acetate–methanol (50:50). This eluate was evaporated to dryness on a rotary evaporator at 40°C and the residue was dissolved in 1 ml methanol. Of this solution, 25 μl were injected into the liquid chromatograph.

HPLC analysis

A Spectra Physics 8100 liquid chromatograph with an SP 8400 UV detector and

an SP 4100 computing integrator was used. The column was a DuPont Zorbax ODS (25 cm × 4.6 mm I.D.) operated at ambient temperature. A Newquard RP-2 precolumn (15 × 3.2 mm I.D.) (Brownlee Labs.) was used to prevent contamination of the analytical column. The mobile phase was methanol–water (90:10) at a flow-rate of 1.0 ml/min. The UV detector was operated at 245 nm. Quantification was performed by comparing sample peak heights with those obtained for standard solutions.

Recovery experiments

The recovery of abamectin from vegetables was determined by applying with a pipette 500-μl aliquots of abamectin standard solutions (54.0 mg/l of Bla and 6.28 mg/l of Blb or 5.40 mg/l of Bla and 0.628 mg/l of Blb) to a 50-g portion of chopped vegetables, which corresponds to 0.54 mg/kg of Bla and 0.0628 mg/kg of Blb or 0.054 mg/kg of Bla and 0.00628 mg/kg of Blb (which is below the limit of detection). To another 50-g portion nothing was added. After a few minutes the samples were analysed as described above. Recoveries were calculated as the difference between the amounts of avermectin Bla or Blb found in the spiked and in the non-spiked samples expressed as a percentage of the amount of avermectin Bla and Blb added.

RESULTS AND DISCUSSION

Fig. 2 shows a typical chromatogram of an abamectin standard solution. We used three different ODS columns: Zorbax ODS, LiChrosorb ODS and Nucleosil ODS. Of these columns, only the Zorbax ODS column yielded a good separation between avermectin Bla and Blb. Fig. 3 and 4 show chromatograms of lettuce extracts with and without abamectin and cucumber extracts with and without abamectin, respectively. The limit of detection, defined as three times the baseline noise was calculated to be 25 ng per injection, which corresponds to 40 μg/kg of avermectin Bla and Blb.

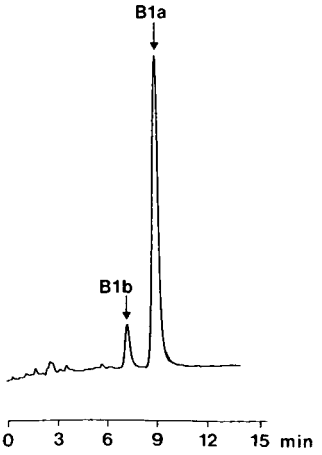

Fig. 2. Chromatogram of abamectine standard. Amounts injected: 810 ng of avermectin Bla and 94.2 ng of avermectin Blb.

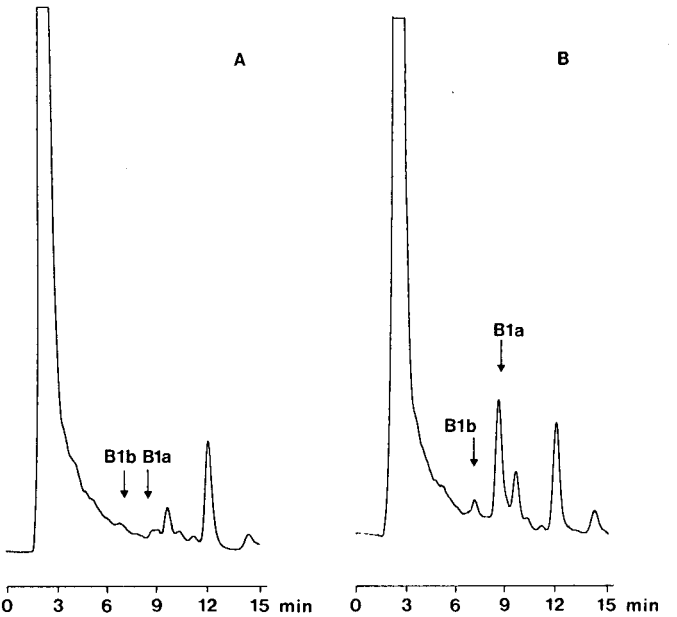

Fig. 3. Chromatograms of (A) unfortified lettuce extract and (B) lettuce extract fortified with 540 μg/kg of avermectin Bla and 62.8 μg/kg of avermectin Blb. Avermectin Bla and Blb peaks represent injected amounts of 337.5 and 39.25 ng of avermectin Bla and Blb, respectively.

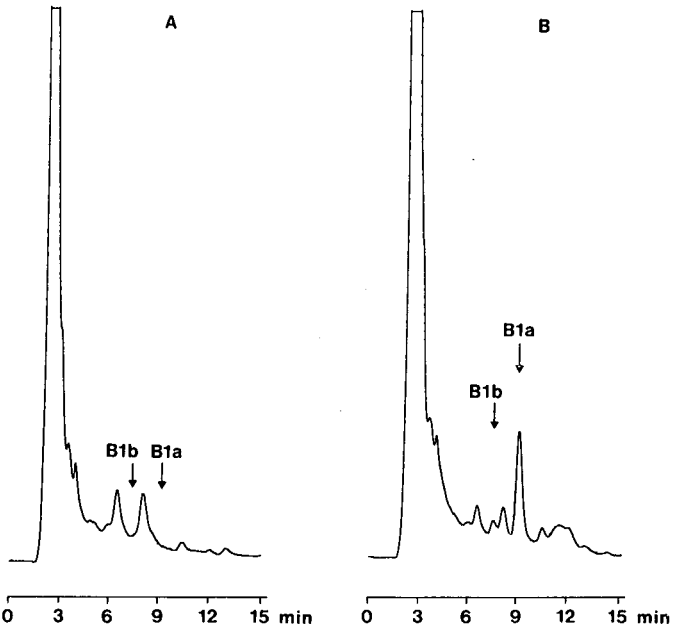

Fig. 4. Chromatograms of (A) unfortified cucumber extract and (B) cucumber extract fortified with 540 μg/kg of avermectin Bla and 62.8 μg/kg of avermectin Blb. Avermectin Bla and Blb peaks represent injected amounts of 337.5 and 39.25 ng of avermectin Bla and Blb, respectively.

TABLE I

RECOVERY STUDY OF ABAMECTIN ADDED TO CHOPPED LETTUCE

Avermectin Bla added (μg/kg)	Amount found (μg/kg)[a]	Recovery (%)	Avermectin Blb added (μg/kg)	Amount found (μg/kg)	Recovery (%)
540	466	86	62.8	54.0	86
540	471	87	62.8	50.0	80
540	458	85	62.8	54.0	86
540	448	83	62.8	54.0	86
540	416	77	62.8	48.0	76
Av. ± S.D.		84 ± 4			83 ± 5
54.0	51.0	94			
54.0	55.0	102			
54.0	59.0	109			
54.0	54.0	100			
54.0	59.0	109			
Av. ± S.D.		103 ± 6			

[a] Non-fortified samples were all below the limit of detection (40 μg/kg).

The mobile phase composition is critical and should be carefully chosen to avoid interfering matrix peaks. We used two Zorbax ODS columns, a new one and one already used for other determinations. On the new column the optimum mobile phase composition was methanol–water (90:10), as described under Experimental. On the old column, a mobile phase composition of methanol–acetonitrile–water (60:30:10) yielded the best separation.

The detector response is linear over a range of at least 25–1350 ng injected

TABLE II

RECOVERY STUDY OF ABAMECTIN ADDED TO CHOPPED CUCUMBER

Avermectin Bla added (μg/kg)	Amount found (μg/kg)[a]	Recovery (%)	Avermectin Blb added (μg/kg)	Amount found (μg/kg)	Recovery (%)
540	473	88	62.8	50.0	80
540	515	95	62.8	50.0	80
540	473	88	62.8	59.0	94
540	437	81	62.8	52.0	83
540	437	81	62.8	52.0	83
Av. ± S.D.		87 ± 6			84 ± 6
54.0	46.0	85			
54.0	46.0	85			
54.0	47.0	87			
54.0	43.0	80			
54.0	47.0	87			
Av. ± S.D.		85 ± 3			

[a] Non-fortified samples were all below the limit of detection (40 μg/kg).

avermectin Bla (correlation coefficient $r = 0.9999$) and 15.7–157 ng injected of avermectin Blb ($r = 0.9999$).

Peaks were identified by comparing the retention times of the peaks on the sample chromatograms with those on the standard chromatograms. The results of a recovery study are given in Tables I and II. The lower fortification level of avermectin Bla in lettuce (Table I) resulted in more than a 100% recovery probably because of a small interfering peak. All other recoveries averaged *ca.* 85%, probably because of some loss in the sample clean-up. Mean recoveries were within the commonly accepted range of 80–110%. The relative standard deviations of the recovery determinations, were not higher than 7%, which is an acceptable precision for residue analysis.

CONCLUSION

Both avermectin Bla and Blb can be detected and determined in lettuce and cucumbers in one chromatogrphic run. The method is rapid, the extraction and clean-up are easy to carry out and derivatization is not necessary. The limit of detection for each component is 40 μg/kg. Clean-up by solid-phase extraction saves time and chemicals and the method should be easily automated with aid of the now commercially available sample pretreatment instruments.

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CHROMSYMP. 2128

Screening for coumatetralyl in soft drinks by solid-matrix extraction and high-performance liquid chromatography with diode-array detection

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ABSTRACT

A method was developed for the rapid determination coumatetralyl in cola- and orange-type soft drinks, which includes extraction using solid-matrix column, clean-up by silica cartridge chromatography and reversed-phase high-performance liquid chromatography with diode-array detection. The recovery of coumatetralyl from 50 ml of soft drinks was better than 80% at spiking levels down to 50 μg/kg (ppb).

INTRODUCTION

Coumatetralyl [CAS Registry No. 5836-29-3; 4-hydroxy-3-(1,2,3,4-tetrahydro-1-naphthalenyl)-2*H*-1-benzopyran-2-one] is an anticoagulant rodenticide with a subchronic LD_{50} (5 days) for rats of 0.3 mg/kg daily [1]. It is used as the active ingredient in numerous commercial rodent baits and concentrates. Recently, we investigated the presence of coumatetralyl in soft drinks in a case of suspected intentional poisoning of beverages from industrial production. A method for rapidly screening for this compound in a large number of samples of orange- and cola-type soft drinks was needed. About 400 samples from different regions of Italy had to be checked by five laboratories in about 2 weeks. The technique of choice for the determination of coumatetralyl is high-performance liquid chromatography (HPLC) [2,3]. However, to our knowledge, no method was available for the extraction and clean-up of coumatetralyl in soft drinks.

Although, the incident with which we had to deal seems unlikely to reoccur, we believe it is useful to report the development of a method for the rapid determination of coumatetralyl in cola-type soft drinks which includes extraction/clean-up on a diatomaceous earth cartridge and determination by reversed-phase HPLC. For orange-type soft drinks a further clean-up by silica gel cartridge chromatography was necessary before HPLC determination.

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EXPERIMENTAL

Reagents

Dichloromethane (analytical-grade reagent), redistilled from an all-glass apparatus, methanol and water (HPLC grade) were used.

Ready-to-use, disposable Chem Elut 2050 cartridges, filled with high-surface-area diatomaceous earth, were obtained from Analytichem International (Harbor City, CA, U.S.A.) and Millipore Sep-Pak silica cartridges from Water Assoc. (Milford, MA, U.S.A.).

Thin-layer chromatographic (TLC) plates, silica gel 60 F_{254}, 5 × 10 cm, with a 0.25-mm layer thickness (E. Merck, Darmstadt, Germany), were used as received.

Apparatus

The HPLC system was composed of a Perkin-Elmer Series 410 pump, a Rheodyne Model 7125 20-μl loop injector and a Perkin-Elmer Model 235 diode-array detector. Spectra were acquired in the range 195–370 nm. The monitoring wavelength in the chromatograms was 285 nm with 0.01 a.u.f.s.

A stainless-steel column (25 cm × 4.6 mm I.D.) packed with Spherisorb S5 ODS-2 (Phase Separations, Queensferry, U.K.) was used, and also a precolumn (4 cm × 2 mm I.D.) dry-packed with Perisorb RP-18, particle size 30–40 μm (E. Merck). The eluent was 0.1% acetic acid–methanol containing 0.1% acetic acid (40:60) at a flow-rate of 1 ml/min.

A rotatory evaporator (bath temperature 40°C; reduced pressure) was used.

Extraction

Transfer 50 ml of cola- or orange-type soft drink into a Chem Elut 2050 cartridge and wait 10 min to allow the liquid to absorb evenly. Add 50 ml of dichloromethane, wait for 3 min, then add two 50-ml portions of dichloromethane. Collect all the dichloromethane eluate and concentrate carefully to dryness. For cola-type soft drinks dissolve the residue in 1 ml of methanol and analyse by HPLC; for orange-type soft drinks, dissolve the residue in 1 ml of dichloromethane and continue with a silica gel cartridge clean-up (see below).

Silica gel cartridge clean-up

Fix a Sep-Pak silica gel cartridge to the outlet of a glass tube (15 cm × 15 mm I.D., terminated with a Luer tip), used as a solvent reservoir. Condition the cartridge with two 2-ml portions of dichloromethane. Transfer the sample solution into the cartridge reservoir and allow it to drain. Apply gentle pressure if the solution does not drain. Carry out two 1-ml washings of the sample vessel with dichloromethane and discard the eluates. Elute coumatetralyl with three 2-ml portions of dichloromethane, collect all the eluates and concentrate carefully to dryness. Dissolve the residue in 1 ml of methanol and analyse by HPLC.

HPLC analysis

Inject 20 μl of the sample into the HPLC system. Using the diode-array detector, compare the retention time and UV spectrum of the standard coumatetralyl with any peak appearing at the same retention time in the chromatogram of the sample.

RESULTS AND DISCUSSION

In the development of the method, the classical separating funnel extraction with dichloromethane was first tried, but was rapidly abandoned because of the formation of intractable emulsions.

In contrast, the solid-matrix extraction of soft drinks with dichloromethane proved to provide a rapid extraction with no emulsion formation; no reusable glassware was needed and the volume of solvent and amount of labour were significantly reduced compared with the separating funnel technique.

The chromatograms of samples of cola-type soft drinks contained some small peaks, but none interfered with the determination of coumatetralyl. Recovery experiments were carried out by spiking "blank" cola samples with standard coumatetralyl. Only one brand of cola was used for the recovery experiments. Samples serving as "blanks" were bought in regions where the batches of suspected poisoned cola had not been sold. These "blank" samples were checked and found to be free from peaks at the retention time of coumatetralyl. The mean recovery ± S.D. (n = 6) was 96 ± 6.5% and 81 ± 8.9% at 200 and 50 μg/kg, respectively. The limit of determination was calculated to be 10 μg/kg.

When orange-type soft drinks were analysed using the same method, they gave complex chromatograms with severe interferences at the retention time of coumatetralyl. It was therefore necessary to clean up these samples further. To this end the chromatographic behaviour of coumatetralyl was studied on non-activated silica gel plates, where coumatetralyl showed approximate R_F values of 0.42, 0.52, and 0.84

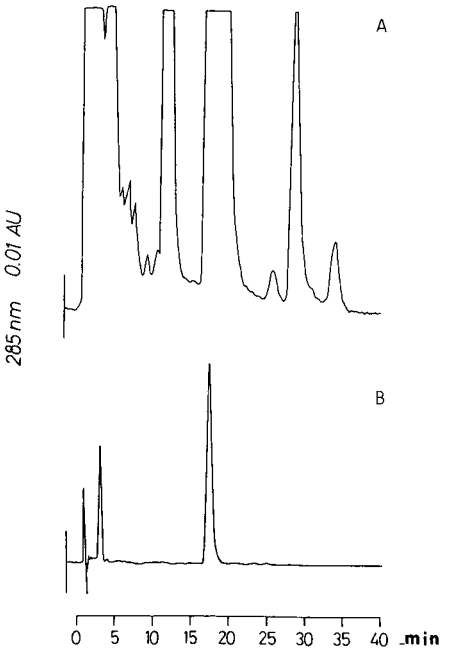

Fig. 1. HPLC of an orange-type soft drink (50 ml) spiked with 200 ppb of coumatetralyl. (A) Crude extract; (B) the same after TLC (wavelength 285 nm; 0.01 a.u.f.s.).

with dichloromethane, dichloromethane + 5% acetone and dichloromethane + 5% methanol, respectively. Therefore dichloromethane was used as the eluent for the separation of coumatetralyl from the raw orange extract obtained by solid-matrix extraction.

Fig. 1 shows the HPLC trace of the raw extract of an orange-type soft drink spiked with 200 ppb (μg/kg) superimposed on the trace obtained by scraping the band eluting on the silica gel TLC plate at the same R_F as the standard coumatetralyl. Thus, the TLC separation was able to remove the interferences and allowed the determination of coumatetralyl. A similar separation was obtained with a Sep-Pak silica gel cartridge, as can be seen in Fig. 2. For orange-type softdrinks spiked with coumatetralyl at 200 and 500 μg/kg levels, the mean recoveries ± S.D. (n = 6) were 92 ± 8.1% and 80 ± 9.2%, respectively, which are of the same order as those obtained with the shorter procedure for cola-type drinks.

The limit of detection (10 μg/kg) is considered to be acceptable for the particular problem concerned, because at this level the daily intake of a 60-kg person drinking 2 l of cola per day would be 0.0003 mg/kg body weight, which is one thousandth of the subchronic LD_{50} (5 days) for rats.

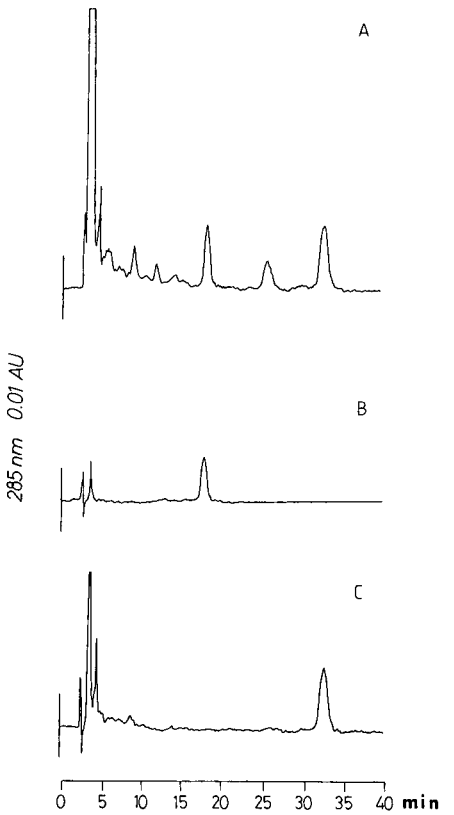

Fig. 2. HPLC of (A) an orange-type soft drink (50 ml) spiked with 50 ppb of coumatetralyl extract after silica gel cartridge clean-up; (B) 15 μg/ml coumatetralyl; (C) reagent blank in the described procedure (wavelength 285 nm; 0.01 a.u.f.s.).

CONCLUSIONS

The method developed offers several interesting features. It makes use of solid-matrix cartridges for extraction and clean-up, which reduces the amount of glassware and solvents needed. The extraction/clean-up procedure requires about 1 h for cola- and 1.5 h for orange-type soft drinks. The use of solid cartridges allows the parallel preparation of several sample extracts. Each HPLC run requires *ca.* 40 min. Hence this method is particularly suited for the screening of large numbers of samples.

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CHROMSYMP. 2219

Evaluation of eluents in thermospray liquid chromatography–mass spectrometry for identification and determination of pesticides in environmental samples

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ABSTRACT

The influence of different eluents in positive and negative ion mode thermospray liquid chromatography–mass spectrometry was studied with several groups of pesticides, including carbamates, chlorotriazines, phenylureas, phenoxy acids and organophosphorus and quaternary ammonium compounds, and the corresponding degradation products. Using the positive ion mode in combination with reversed-phase eluents the base peaks generally corresponded either to $[M + H]^+$ for the chlorotriazines and their hydroxy metabolites or to $[M + NH_4]^+$ for the carbamates, the phenylureas, the organophosphorus pesticides and their oxygen analogues. In the negative ion mode different processes such as (dissociative) electron-capture and anion attachment mechanisms occurred. Fragment ions such as $[M - CONHCH_3]^-$ for the carbamates, $[M - H]^-$ for the chlorotriazines, phenylureas and chlorinated phenoxy acids and $[M]^{\cdot -}$, $[M - R]^-$ (R being a methyl or ethyl group) for organophosphorus pesticides were usually formed. Depending on the eluent additive used (ammonium acetate, ammonium formate and/or chloroacetonitrile), three different adduct ions were formed: $[M + CH_3COO]^-$, $[M + HCOO]^-$ and $[M + Cl]^-$.

Normal-phase eluents with cyclohexane, *n*-hexane and/or dichloromethane provided more structural information and enhanced the response of several compounds. The positive ion mode was useful for the detection of chlorinated phenoxy acids and chlorophenols which could not be detected in the positive ion mode using reversed-phase systems. The base peaks generally corresponded to $[M]^{\cdot +}$, $[M + H]^+$ or $[M - Cl]^+$.

For the characterization of difenzoquat, a quaternary ammonium pesticide of which trace level analysis is troublesome, a post-column ion-pair extraction system was used. An aqueous mobile phase with a sulphonate-type counter ion was applied and an extraction solvent containing cyclohexane–dichloromethane–*n*-butanol (45:45:10) was used in thermospray liquid chromatography–mass spectrometry. Illustrative examples of the determination of residue levels of pesticides in soil matrices are shown.

INTRODUCTION

The determination of thermally labile and/or polar pesticides, which is troublesome by gas chromatography(GC)–mass spectrometry (MS), can be partly achieved

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by using liquid chromatography (LC)–MS. During the last decade, LC–MS with different interfaces such as the moving belt, direct liquid introduction (DLI), thermospray (TSP) and particle beam, has developed into a well established technique with practical applications for characterizing different groups of pesticides, *e.g.*, triazines [1–4], organophosphorus pesticides [5–7], chlorinated phenoxy acids [2,3,8], phenylureas [2,7,9] and carbamates [10–12]. Recently, two monographs [13,14] have been published on the use of LC–MS systems in pesticide analysis.

Among the different interfaces, TSP is the most popular nowadays in reversed-phase (RP) where acetonitrile–water or methanol–water mixtures containing a volatile buffer (ammonium acetate) are used as the eluent. One of the drawbacks of TSP-LC–MS is the lack of structural information that can be obtained. Normally the positive ion (PI) mode is used and the only ions which are usually formed are the protonated molecular ion $[M+H]^+$ and/or an adduct ion $[M+NH_4]^+$. Different approaches have been described for improving fragmentation, such as the use of the PI and the negative ion (NI) mode [6,7,15], the use of ammonium acetate, ammonium formate or chloroacetonitrile as eluent additives [2,8,15] and normal-phase (NP) eluents (*e.g.*, cyclohexane [3,16]). As instrumental procedures to induce fragmentation, the use of a repeller electrode operated at high voltage comparable to collision-induced dissociation (CID) [17] and CID TSP-LC–MS–MS [18] has been reported. These techniques have been demonstrated to provide adequate information for the identification and determination of pesticides in environmental samples.

The objective of this work was to study the potential of obtaining more structural information in filament-on TSP-LC–MS for the identification and determination of pesticides in environmental samples. Case studies are used to describe the possibilities for organophosphorus pesticides and their oxygen analogues, carbamates and their corresponding hydroxypirimidine metabolites, chlorotriazines and hydroxytriazines, chlorinated phenoxy acids, chlorophenols and phenylurea herbicides. A post-column extraction system, equipped with a sandwich phase separator, for the ion-suppressed analysis of chlorinated phenoxy acids and the ion-pair extraction of quaternary ammonium pesticides will be described.

EXPERIMENTAL

Chemicals

High-performance liquid chromatographic-grade water from Riedel-de Haën (Seelze-Hannover, Germany) and methanol, acetonitrile and chloroacetonitrile from Merck (Darmstadt, Germany) were passed through a 0.45-μm filter (Scharlau, Barcelona, Spain) before use. Acetone, ethyl acetate, *n*-hexane, diethyl ether and dichloromethane were of pesticide grade, obtained from SDS (Peypin, France). Analytical-reagent grade carbaryl, oxamyl, atrazine, simazine, cyanazine, fonofos, fensulfothion, trichlorfon, parathion-ethyl, phosmet, chlorpyrifos, (2,4-dichlorophenoxy)-acetic acid (2,4-D), (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T), (2,4,5-trichlorophenoxy)propionic acid (Silvex), monuron and diuron were purchased from Polyscience (Niles, IL, U.S.A.), difenzoquat from Promochem (Wesel, Germany) and linuron from Riedel-de Haën. Fenitrothion was a gift from Sumitomo (Osaka, Japan) and fenitrooxon was synthesized [19]. Florisil (100–200 mesh) was purchased from Merck. Ammonium acetate and ammonium formate were supplied by Panreac (Barcelona, Spain) and Fluka (Buchs, Switzerland), respectively.

Sample preparation

Sample pretreatment for the determination of organophosphorus and phenylurea pesticides in soil was carried out using a modification of the procedure reported by Durand *et al.* [20]. The procedure includes freeze-drying of soil samples, filtering (120 μm) and Soxhlet extraction for 12 h with methanol. After concentration to *ca.* 20–25 ml in a rotary evaporator (35°C), the extract was carefully evaporated to dryness. Subsequently, the residue was dissolved in *n*-hexane and cleaned up on Florisil with elution using diethyl ether–*n*-hexane (50:50). Finally, methanol was added to a final volume of 0.5 ml and 20 μl of this solution were injected into the LC–MS system.

Chromatographic conditions

Chromatography was performed with two Model 510 high-pressure pumps coupled with a Model 680 automated gradient controller (Waters Chromatography Division, Millipore, Bedford, MA, U.S.A.) and a Model 7125 injection valve with a 20-μl loop from Rheodyne (Cotati, CA, U.S.A.). LiChrocart cartridge columns (12.5 cm × 4.0 mm I.D.) packed with 5-μm LiChrospher 100 RP-18 from Merck were used. The following eluents were tested: methanol–water (50:50) containing 0.05 *M* ammonium acetate, methanol–water (50:50) + 0.05 *M* ammonium formate, acetonitrile–water (50:50) containing 0.05 *M* ammonium formate and acetonitrile–water–chloroacetonitrile (49:49:2) containing 0.05 *M* ammonium acetate at a flow-rate of 1 ml/min. Cyclohexane and *n*-hexane were used at 0.7–0.8 ml/min. All solvent compositions are represented volume-to-volume.

Mass spectrometric analysis

A Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 5988A TSP-LC–MS quadrupole mass spectrometer and a Hewlett-Packard Model 59970C instrument for data acquisition and processing were employed. For most of the RP-LC conditions, the temperatures of the TSP system were 100, 188 and 270°C for the stem, tip and vapour and ion source, respectively. Some experiments were carried out at a vapour and ion source temperature of 200°C, keeping the other temperatures as above. When NP or post-column extraction systems were used, the temperatures of the TSP system were 86, 188 and 200°C, respectively. In all the experiments, the filament was on.

RESULTS AND DISCUSSION

Positive and negative ion modes

In most TSP-LC–MS systems methanol–water or acetonitrile–water mixtures containing a volatile buffer (ammonium acetate) are used to enhance ionization in the PI mode resulting in the formation of combinations of $[M+H]^+$ and $[M+NH_4]^+$ ions as base peaks. As a result, relatively simple mass spectra are obtained, which means that this mode is nowadays used for routine analysis.

The NI mode, however, is not as frequently applied as the PI mode, probably because the response is much more compound dependent than in the PI mode, although the mechanisms involved such as anion attachment and proton abstraction have been pointed out to be predominant [21] and similar to conventional negative chemical ionization (NCI) and DLI-LC–MS. These processes, *e.g.*, resonance and dissociative electron capture, proton transfer and anion attachment, and parameters

TABLE I

IMPORTANT FRAGMENTS AND RELATIVE INTENSITIES OBSERVED UNDER RP-TSP-LC–MS CONDITIONS

Carrier stream: methanol–water or acetonitrile–water* with different eluent additives (A = 0.05 *M* ammonium acetate, B = 0.05 *M* ammonium formate, C = 2% chloroacetonitrile). Filament-on mode of operation.

Mol. wt.	Compounds and ions (m/z and tentative identification)		A	B	C
Carbamate pesticides					
201	Carbaryl				
	202	$[M+H]^+$	10	10	n.i.[a]
	203	$[M-CONHCH_3+CH_3COOH]^-$	100		
	219	$[M+NH_4]^+$	100	100	
	260	$[M+CH_3NH_2CO]^+$	15		
219	Oxamyl				
	193	$[M-CON(CH_3)_2+HCOO]^-$		100	n.i.
	220	$[M+H]^+$	10	5	
	237	$[M+NH_4]^+$	100	100	
*Chlorinated phenoxy acids**					
220	2,4-D				
	219	$[M-H]^-$	30	30	10
	255	$[M+Cl]^-$	2		100
	265	$[M+HCOO]^-$		100	
	279	$[M+CH_3COO]^-$	100		10
	315	$[M+(CH_3COOH)\cdot Cl]^-$			10
268	Silvex				
	267	$[M-H]^-$	40	40	10
	269	$[M+H]^-$	50	50	7
	303	$[M+Cl]^-$	5	5	50
	313	$[M+HCOO]^-$		100	2
	327	$[M+CH_3COO]^-$	100		2
Chlorotriazine herbicides					
215	Atrazine				
	216	$[M+H]^+$	100	100	n.i.
	257	$[M+CH_3CN+H]^+$	30*	70*	
	275	$[M+CH_3COONH_4+H-H_2O]^+$	20		
240	Cyanazine				
	240	$[M]^{\cdot-}$	100	100	n.i.
	241	$[M+H]^+$	100	100	
	275	$[M+Cl]^-$	60	60	
	282	$[M+CH_3CH+H]^+$	25*	25*	
Organophosphorus pesticides					
256	Trichlorfon				
	205	$[M-HCl-CH_3]^-$	40	40	
	241	$[M-CH_3]^-$	20	20	7
	291	$[M+Cl]^-$	100	100	100
	301	$[M+HCOO]^-$		20	
	315	$[M+CH_3COO]^-$	20		
	351	$[M+(CH_3COOH)\cdot Cl]^-$			10

TABLE I *(continued)*

Mol. wt.	Compounds and ions (m/z and tentative identification)		A	B	C
277	Fenitrothion				
	141	$[FG]^-$	15	15	n.i.
	262	$[M-CH_3]^-$	15	15	
	277	$[M]^{\cdot -}$	100	100	
	278	$[M+H]^+$	30	30	
	295	$[M+NH_4]^+$	100	100	
Phenylurea herbicides					
198	Monuron				
	199	$[M+H]^+$	30	30	n.i.
	216	$[M+NH_4]^+$	100	100	
	233	$[M+Cl]^-$	15	15	
	243	$[M+HCOO]^-$		100	
	257	$[M+CH_3COO]^-$	100		
	275	$[M+CH_3COONH_4+NH_4-H_2O]^+$		50	
248	Linuron				
	247	$[M-H]^-$	100	100	n.i.
	249	$[M+H]^+$	20	20	
	266	$[M+NH_4]^+$	100	100	
	283	$[M+Cl]^-$	80	80	
	307	$[M+CH_3COONH_4+NH_4-H_2O]^+$		40	

[a] Not investigated.

such as pressure and temperature, impurities and radical species in the reagent gas and source characteristics, are involved in the ion formation in the NI mode of TSP-LC–MS.

When comparing the PI and NI modes for the different groups of pesticides, both qualitative and quantitative aspects should be considered. With respect to structure identification, the similarity between the TSP-LC–MS results in the PI and in the NI mode should be studied.

Table I shows the main ions obtained in the PI and NI modes for selected pesticides of different chemical groups and with the equipment described under Experimental. In the NI mode the ions formed depended on the compound and eluent used, whereas in the PI mode the different groups of pesticides behaved more similarly.

From Table I it can be seen that in the PI mode the $[M+H]^+$ (for chlorotriazines) and the $[M+NH_4]^+$ (for carbamates, organophosphorus pesticides, phenylurea herbicides) ions are the base peaks in the TSP mass spectra. Exceptions are those compounds having a higher proton affinity than the ammonium ion, as a result of their pyrimidinyl, amidate and pyrimidine structure, such as the organophosphorus pesticides, diazinon or fenamiphos [22,23], and the carbamates, pirimicarb and their pyrimidine metabolites [12], which exhibit $[M+H]^+$ as the base peak. The phenoxy acids and their degradation products, the chlorophenols, are hardly ionized in the PI mode because of the relative high electronegativity of these solutes. Because the quaternary ammonium compounds are already ionized, the M^+ ion is the base peak.

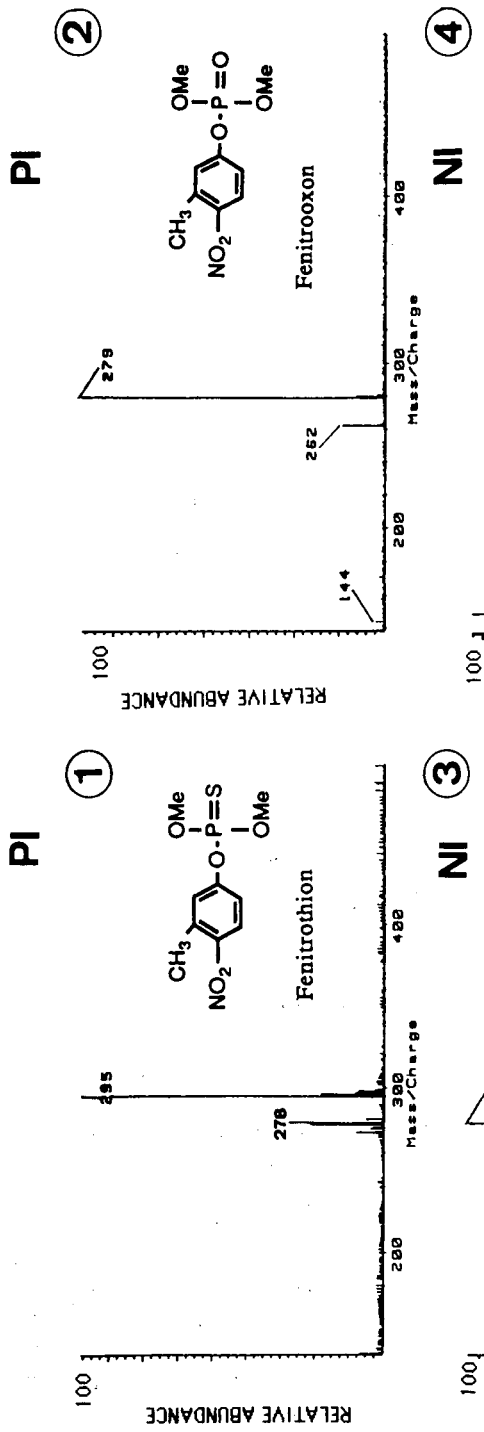

Fig. 1. Flow injection TSP mass spectra in the PI (spectra 1 and 2) and NI (spectra 3 and 4) modes for fenitrothion and fenitrooxon. Amount injected, *ca.* 1 μg; carrier stream, methanol–water (50:50) + 0.05 *M* ammonium formate; flow-rate, 1 ml/min; TSP vapour and ion source temperature, 270°C. Me = Methyl.

In the NI mode, the formation of anions depends on the chemical structure of the pesticide. For most of the carbamates, hardly any signal in the NI mode was obtained when 1 μg of sample was injected. Carbamates such as carbaryl and oxamyl exhibited anions like $[M-CONHCH_3]^-$, $[M-CONHCH_3+CH_3COOH]^-$ and $[M-CON(CH_3)_2+CH_3COOH]^-$, which correspond to the cleavage of the alkyl side-chain or the aromatic ring, just as observed in DLI-LC–MS [24]. For cyanazine, a chlorotriazine herbicide, $[M]^{\cdot -}$ was normally obtained as the base peak in combination with the chloride attachment $[M+Cl]^-$ ion, as a second abundant ion [12]. Typical NCI mechanisms were observed for the organophosphorus pesticides. The specific fragments were $[M-R]^-$, R being methyl or ethyl, $[FG]^-$ (FG being the functional group fragment of the organophosphorus pesticide), $[M]^{\cdot -}$, $[M-Cl]^-$, the phenolate and thiophenolate anions and adducts with the ionizing additive [6,7,25]. For the phenylurea herbicides, $[M-H]^-$ and $[M+CH_3COO]^-$ ions were, in general, the base peaks, while $[M+Cl]^-$ was a minor ion [7,26]. Finally, for the chlorinated phenoxy acids, anions such as $[M-H]^-$, $[M+H]^-$, $[M+CH_3COO]^-$ and $[M+Cl]^-$ were obtained. In this instance the base peak depended strongly on the eluent used [2,3,8,16].

In Fig. 1 an example is shown of the information that can be obtained by using both the PI and NI modes. The TSP mass spectra of fenitrothion and fenitrooxon are shown. The base peaks of fenitrothion and fenitrooxon in the PI mode correspond to the $[M+NH_4]^+$ ion with *m/z* values of 295 and 279, respectively. Other ions correspond to the $[M+H]^+$ ion at *m/z* 278 for fenitrothion and at *m/z* 262 for fenitrooxon. Some of the fragment ions observed for fenitrothion in NI-TSP–MS are similar to those reported in NCI-MS [27] such as fragment ions at *m/z* 141, 262 and 277 corresponding to the $[FG]^-$, $[M-CH_3]^-$ and $[M]^{\cdot -}$ ions, respectively. With fenitrooxon, the phenolate anion at *m/z* 152, corresponding to $[OC_7H_6NO_2]^-$, is the base peak. The second abundant ion corresponds to $[M]^{\cdot -}$ at *m/z* 261. Hence, by using both

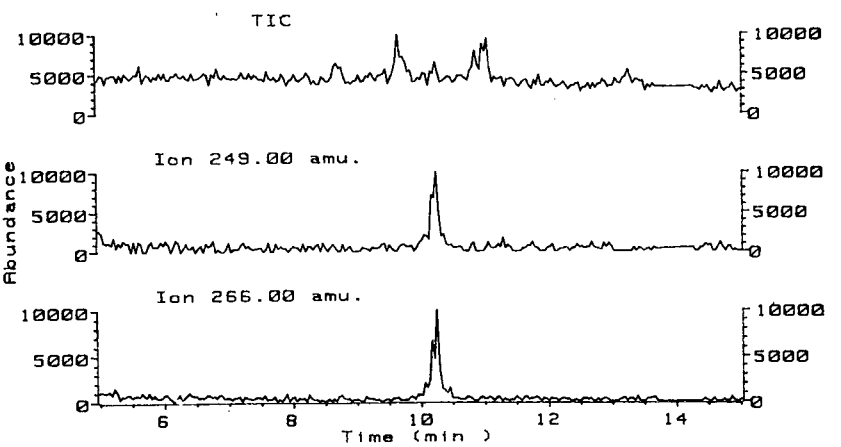

Fig. 2. Total ion current and selected ion chromatograms in PI mode for a real soil sample from the Ebro Delta containing 0.5 μg/g of linuron, collected 1 month after pesticide application. Monitored ions correspond to $[M+H]^+$ and $[M+NH_4]^+$, with *m/z* values of 249 an 266, respectively. Mobile phase, methanol–water (80:20) + 0.05 *M* ammonium formate; flow-rate, 1 ml/min; TSP vapour and ion source temperature, 270°C.

modes of operation, a complete and unequivocal identification of both organophosphorus pesticides is achieved.

In Fig. 2 the total ion current and the selected ion chromatograms are shown for a soil sample polluted with 0.5 μg/g of linuron, applied as a herbicide 1 month before the sample was taken. The presence of linuron is clearly shown in the sediment sample.

The second aspect, in comparing the PI and NI modes in TSP-LC–MS, is the quantitative potential of each mode. In Table II, the detection limits using RP eluents for the various groups of pesticides are shown. In general, the PI mode is recommended for the analysis of compounds with proton affinity values higher than or close to that of ammonia, such as carbamate insecticides and chlorotriazine and phenylurea herbicides. For the organophosphorus pesticides from the parathion group (phosphorothionates with an aromatic structure like parathion), although they also exhibit proton affinity values close to ammonia, they can easily stabilize the negative charge under NI conditions, owing to the chemical structure. Hence, the sensitivities for the PI and the NI modes are approximately identical. With the oxygen analogues, because the oxo group enhances the proton affinity in comparison with the thio group, the sensitivity is one order of magnitude better in PI compared with the NI mode. For the chlorinated phenoxy acids and chlorophenols the sensitivity in the NI mode is much better than that in PI mode. This is due to their proton affinity values, which are much lower than that of ammonia, so no signal is obtained under PI conditions.

TABLE II

DETECTION LIMITS (FULL SCAN) FOR THE DIFFERENT GROUPS OF PESTICIDES WITH TSP-LC–MS

LC eluent: methanol–water (50:50) or (see footnote) acetonitrile–water (50:50) + 0.05 *M* ammonium acetate or 0.05 *M* ammonium formate

Pesticides	Detection limit (ng)	
	PI	NI
Organophosphorus pesticides (parathion group)	20–50	50–70
Organophosphorus pesticides (oxygen analogues) (paraoxon group)	1–2	50–70
Carbamates (in general)	1–2	>200
Hydroxypyrimidine and pirimicarb metabolites	1–2	1–2
Chlorotriazines + hydroxytriazines (except cyanazine)	5–10	100
Chlorinated phenoxy acids[a]	>200[a]	1–10
Chlorophenols[a]	>200[a]	1–10
Phenylurea herbicides	2–5	10–20
Quaternary ammonium compounds[a] (only difenzoquat)	~100	n.m.[b]

[a] Acetonitrile–water (50:50) eluent.
[b] Not measured.

Influence of (ionizing) additives in TSP-LC–MS eluents

The lack of structural information observed for some of the tested pesticide groups is the main limitation of TSP-LC–MS. A possible solution is the use of eluent additives to form adducts which provide more information for molecular weight assignment and/or structure identification. In the PI mode the formation of adduct ions in TSP-LC–MS follows a general rule as was recently proposed by Maeder [28]. The masses of these adduct ions can be described by the equation

$$M_{\text{adduct}} = [M + A + xB - yH_2O]^+ \quad (1)$$

where M is the molecular weight of the analyte, A is the attached ion (H^+ or NH_4^+), B is the attached molecule (CH_3OH, H_2O, CH_3CN) and x and y can have the values 0, 1, 2, etc.

Changing the eluent from acetonitrile–water to methanol–water or using ammonium formate or ammonium acetate as ionizing additives will not change the base peaks of the different pesticides. Significant differences are observed, however, for the other fragment ions. The $[M + CH_3CN + H]^+$ and $[M + CH_3CN + NH_4]^+$ ions showed relative abundances of 40–70% for the carbamates and the chlorotriazines when acetonitrile–water mixtures were used [12]. When ammonium acetate was used as an ionizing additive with acetonitrile–water or methanol–water mixtures, adduct ions such as $[M + CH_3COONH_4 + NH_4^+ - 2H_2O]^+$ or $[M + CH_3COONH_4^+ + H - H_2O]^+$ appeared in the spectra. Although the base peak was normally the same, for the chlorotriazines different base peaks were observed when the vapour temperature of the TSP interface was changed. At 200°C the $[M + CH_3COONH_4 + H - H_2O]^+$ ion was the base peak using ammonium acetate as ionizing additive [2], but at 270°C the $[M + H]^+$ ion was the base peak, because of the thermally induced dissociation in the ion source.

In the NI mode, different adduct ions have been reported for a given additive

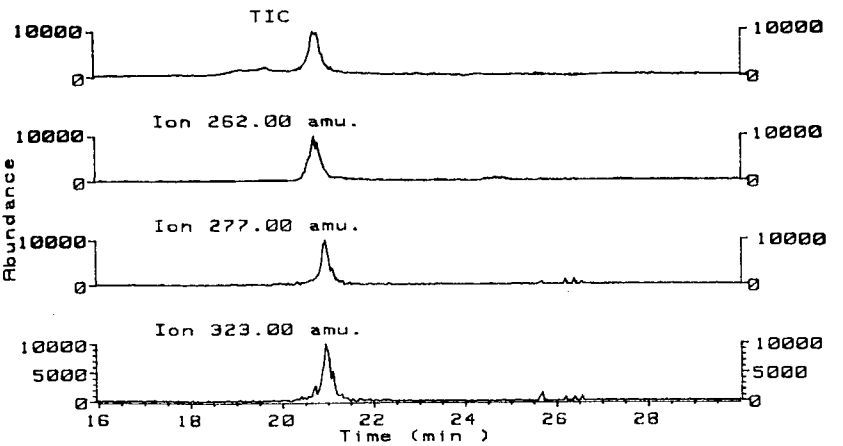

Fig. 3. Total ion current and selected ion chromatograms in NI mode for a real soil sample from the Ebro Delta containing 0.8 μg/g of fenitrothion, collected 2 days after aircraft application. Ions monitored correspond to $[M - CH_3]^-$, $[M]^{\cdot -}$ and $[M + HCOOH]^-$, with m/z values of 262, 277 and 323, respectively. Chromatographic conditions as in Fig. 2; TSP vapour and ion source temperature, 200°C.

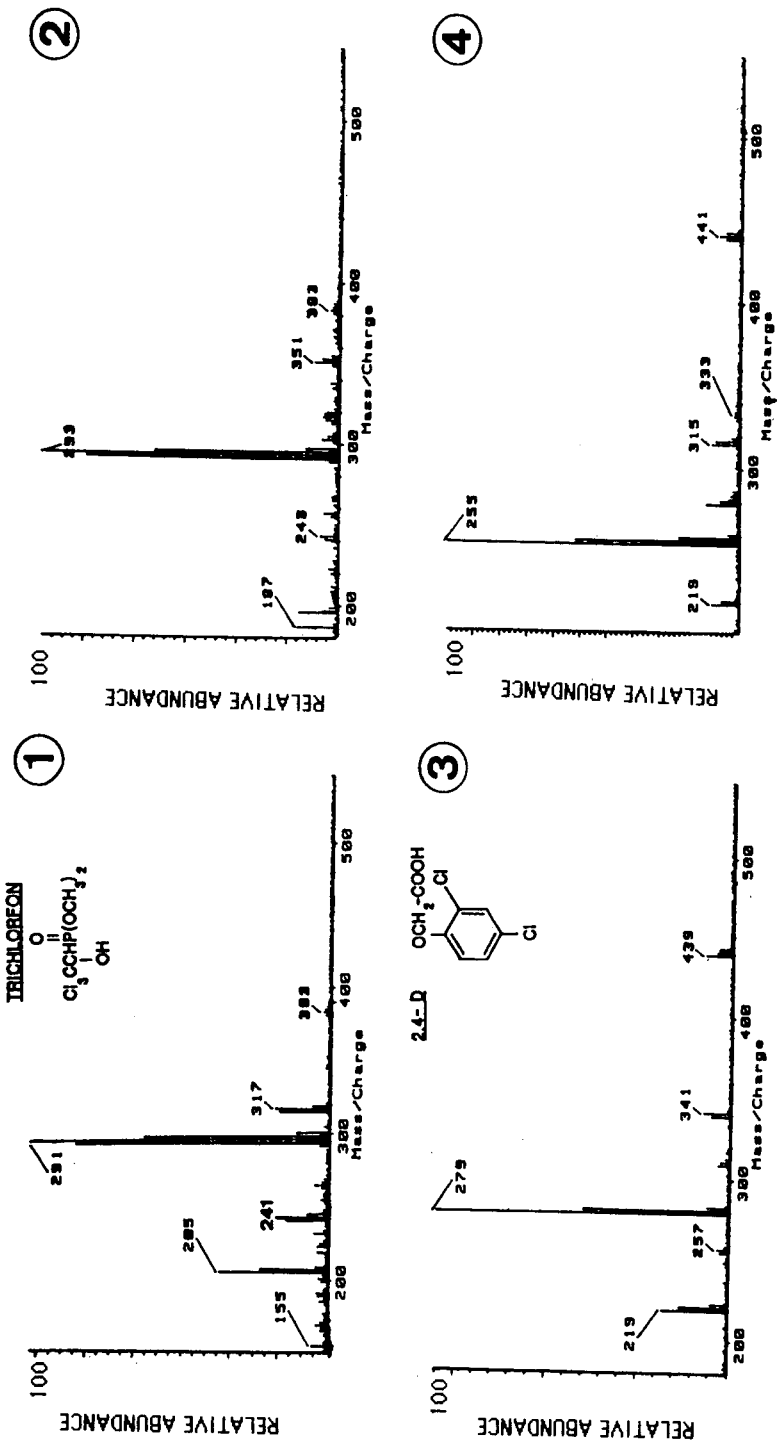

Fig. 4. Flow-injection NI mode TSP-MS spectra of trichlorfon (spectra 1 and 2) and 2,4-D (spectra 3 and 4). Carrier stream, (1, 3) acetonitrile–water (50:50) + 0.05 *M* ammonium acetate and (2,4) acetonitrile–water–chloroacetonitrile (49:49:2) + 0.05 *M* ammonium acetate; flow-rate, 1 ml/min; amount injected, 1 μg; TSP vapour and ion source temperature, 200°C.

depending on the eluent. Because ammonium acetate is the most frequently applied buffer, the $[M + CH_3COO]^-$ ion is the most cited adduct ion. Adducts with formate have also been reported [2,12,15] to yield additional molecular weight information. An example is the analysis of a soil sample from the Ebro Delta (Tarragona, Spain), where fenitrothion is currently applied as an insecticide [29] (Fig. 3). The $[M - R]^-$ anion, in this instance the $[M - CH_3]^-$ ion, seems to be typical of the organophosphorus pesticides of the parathion group. A third ion, corresponding to $[M + HCOOH]^-$, was also used in this instance for confirmation purposes and it is an attachment ion obtained in this case and not in the spectra in Fig. 1. This has been previously explained for this compound in NI-TSP–MS and it is due to the fact that a lower vapour temperature (200°C in Fig. 3 *versus* 270°C in Fig. 1) produces higher abundances of anion attachment ions [25]. These ions provide adequate proof of the presence of fenitrothion in sediment. In PI TSP-LC–MS only two ions, corresponding to the $[M + H]^+$ and $[M + NH_4]^+$ ions, were observed, so less structural information was obtained than in the NI mode.

The applicability of chloride-attachment phenomena for obtaining complementary structural information using chloracetonitrile in the eluent has proved to give satisfactory results for chlorine-containing compounds [8]. In the NI mode, the mentioned additives (ammonium acetate, ammonium formate and chloracetonitrile) all provide additional adduct ion information for compounds with a good signal in the NI mode, such as chlorinated phenoxy acids and chlorophenols [2,3,8,15]. As an example, the TSP mass spectra of trichlorfon and 2,4-D are given in Fig. 4. A mixture of acetonitrile and water, containing ammonium acetate as the buffer, was used with and without the addition of 2% chloroacetonitrile. For trichlorfon, an organophosphorus pesticide containing a chlorine atom, the $[M + Cl]^-$ ion (*m/z* 291) is already the base peak even without the addition of chloracetonitrile. This ion can be due to self-attachment of chlorine to the molecule by pyrolysis in the TSP aerosol of this thermally labile compound [6]. The other anions found for trichlorfon which have also been reported in NCI DLI-LC–MS [5] corresponded to $[M - HCl - CH_3]^-$, $[M - CH_3]^-$ and $[M + CH_3COO]^-$ at *m/z* 205, 241 and 315, respectively.

For 2,4-D, the base peak without the addition of chloracetonitrile corresponds to $[M + CH_3COO]^-$. As the molecule also contains a chlorine atom, a chloride attachment ion at *m/z* 255 was observed, but because 2,4-D itself is less thermolabile than trichlorfon this chloride attachment peak was not the base peak. Adding chloroacetonitrile to the eluent changed both spectra. For trichlorfon, the base peak remained the same, but the other ions nearly disappeared, indicating that only chloride attachment was taking place. For 2,4-D the new base peak was the $[M + Cl]^-$ ion and the other ions were relatively weak, as in the case of trichlorfon. Additional chlorine anion attachment ions at *m/z* 351 and 315 for trichlorfon and 2,4-D, respectively, are obtained when chloroacetonitrile is added to the eluent, which corresponds to the $[M + (CH_3COOH) \cdot Cl]^-$ ion, as reported previously for 2,4-D [8].

Normal-phase eluents

In most instances RP eluents are used in TSP-LC–MS and only a few applications have been reported using NP eluents such as *n*-hexane, cyclohexane or dichloromethane [3,16]. The necessity for using NP-LC for a number of analyses results in a wider application range of TSP-LC–MS. Moreover, the detection possibilities of

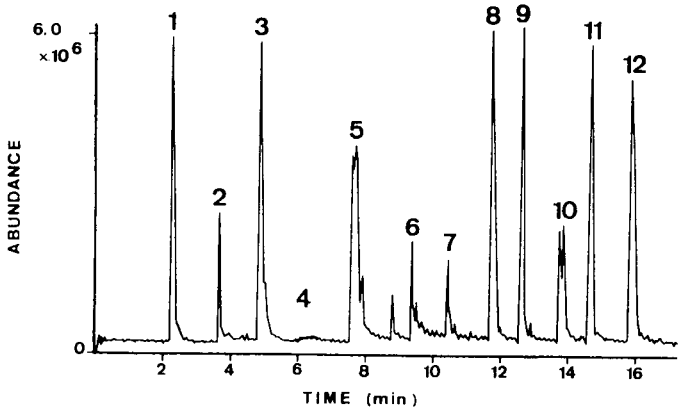

Fig. 5. Flow-injection PI mode TSP-MS reconstructed ion current of several pesticides and chlorophenols (*ca.* 500 ng each) using cyclohexane as carrier stream. Compounds: 1 = 2,4-dichlorophenol; 2 = Silvex; 3 = trichlorfon; 4 = pentachlorphenol; 5 = phosmet; 6 = 2,4-D; 7 = 2,4,5-T; 8 = parathion-ethyl; 9 = 2,4,5-trichlorophenol; 10 = fensulfothion; 11 = fonofos; 12 = chlorpyrifos. Flow-rate, 0.7 ml/min; TSP vapour and ion source temperature, 200°C.

TSP-LC–MS will be improved, as additional structural information and/or greater sensitivity can be obtained.

The main advantage of NP eluents in TSP-LC–MS is the improved response in this mode for compounds with high electronegativity, *e.g.*, chlorophenols and chlorinated phenoxy acids. The detection limits for these compounds in the NI and PI modes, using an NP eluent, were 1–10 and 2–20 ng, respectively. The total ion current trace of individual test compounds (organophosphorus pesticides, chlorophenols and chlorinated phenoxy acids) injected one after the other in the PI-TSP-MS system is shown in Fig. 5. Cyclohexane was used as the carrier stream and reagent gas. The chlorophenols and chlorinated phenoxy acids did not show any response when the same amount was injected as in an RP eluent using the PI mode.

In Fig. 6 the PI and NI-TSP mass spectra of chlorpyrifos in cyclohexane and parathion-ethyl and 2,4,5-T in dichloromethane are shown. The observed base peaks in both modes can be explained by the similarity of the chemical ionization process in TSP-LC–MS and DLI-LC–MS. This similar behaviour was even more pronounced when TSP operation was carried out without a buffer and with the filament on. In this case $[M+NH_4]^+$ ions were not formed at all. When the different PI-TSP spectra in Fig. 6 are compared, it is obvious that the base peak was the $[M+H]^+$ ions for the organophosphorus pesticides, chlorpyrifos and parathion-ethyl, and the $[M]^{\cdot+}$ ion for 2,4,5-T. In PI-DLI with RP eluents normally the $[M+H]^+$ ion is the base peak for the organophosphorus pesticides [5] and the $[M]^{\cdot+}$ ion is observed for chlorinated phenoxyacids, with relative intensities of *ca.* 10% [24].

Further, it can be seen that the NI-TSP spectra offer significantly more structural information than PI-TSP spectra. In NI-TSP-MS the spectra were similar to those obtained by conventional NCI-MS and DLI-LC–MS using the NI mode. Chemical ionization processes such as dissociative electron capture, resonance electron capture, proton transfer and anion attachment occur. For chlorpyrifos, most of the ions were

observed in NCl-MS. The ions observed were the functional group fragment of the organophosphorus pesticide $[FG]^-$, $[M-(C_2H_5O)_2PO]^-$, $[M-HCl]^-$ and $[M]^{\bullet -}$. They had relative abundances of 48, 61, 100 and 12%, respectively, in NCl–MS [27], which is consistent with the TSP mass spectra in Fig. 6. For parathion-ethyl, the base peaks in NCl-MS or DLI-LC–MS [30] corresponded to the thiophenolate anion at m/z 154, whereas the other ions exhibited relative abundances below 30%. In TSP-LC–MS the abundance of the ions was not the same as in DLI-LC–MS or NCl-MS. Moreover, the base peak was the $[M-R]^-$ ion (at m/z 262) and about 15% was the $[M-H+Cl]^-$ ion, corresponding to a mixed mechanism of proton abstraction and chloride attachment. The $[M-R]^-$ ion was also observed as the base peak for most of the organophosphorus pesticides when 1% chloroacetonitrile was added to an RP eluent in DLI-LC–MS [5]. The 15% relative abundance of the chloride adduct was due to the use of dichloromethane as reagent gas, which favours the addition of a chlorine atom. For 2,4,5-T, the base peak was the $[M+Cl]^-$ ion, at $m/z = 289$. This can be explained by the relatively high concentration of chlorine available in the ion source available from the dichloromethane combined with the pyrolytic behaviour of the analyte itself, in a similar manner to that mentioned previously for aromatic chlorine-containing compounds in NI-TSP-LC–MS [8,15,21,26]. The $[M+Cl]^-$ ion has previously been observed in DLI-LC–MS [31], with abundances of up to 60% with RP systems in TSP-LC–MS [2,3,8]. Abundances up to 100% were found when 2% chloracetonitrile was added to an RP-LC eluent [8,15].

Post-column extraction strategies

In TSP-LC–MS, normally RP eluents containing volatile buffers (*e.g.*, ammonium acetate or ammonium formate) are used. However, in many pesticide analyses non-volatile buffers and/or ion-pairing reagents are applied. The latter system can be combined with TSP-LC–MS using a post-column extraction system. The applicability of this system is demonstrated with the ion-suppressed liquid–liquid extraction of chlorinated phenoxy acids and with the ion-pair extraction of difenzoquat, one of the quaternary ammonium compounds used as a herbicide nowadays. A schematic diagram of the experimental set-up has been described [16]. The phase separator in this system allows an efficient separation of an organic and an aqueous phase, so a purely organic phase can be obtained which can be directly introduced into the mass spectrometer by means of the TSP interface. The flow of the organic solvent is regulated by a PTFE capillary and a restrictor. For the ion-suppressed extraction of chlorinated phenoxy acids, an RP-LC separation on a C_{18} column is applied with an eluent consisting of acetonitrile–water (50:50) containing 0.1 *M* phosphate buffer (pH 2.5), ensuring that the acids were non-dissociated, at a flow-rate of 1 ml/min. The post-column extraction was performed with dichloromethane–cyclohexane–*n*-butanol (45:45:10) at a flow-rate of 1 ml/min. In this way the chlorinated phenoxy acids were extracted into the organic phase and subsequently introduced into the TSP-LC–MS system. The extraction efficiencies for 2,4-D, 2,4,5-T and Silvex were 100%, 70% and 60%, respectively.

The analysis of quaternary ammonium compounds is tedious and involves either multi-step extraction and derivatization processes prior to GC determination [32,33] or cation-exchange LC and UV detection [32,34,35]. Owing to problems with separation and detection [32], methods based on fast atom bombardment MS–MS

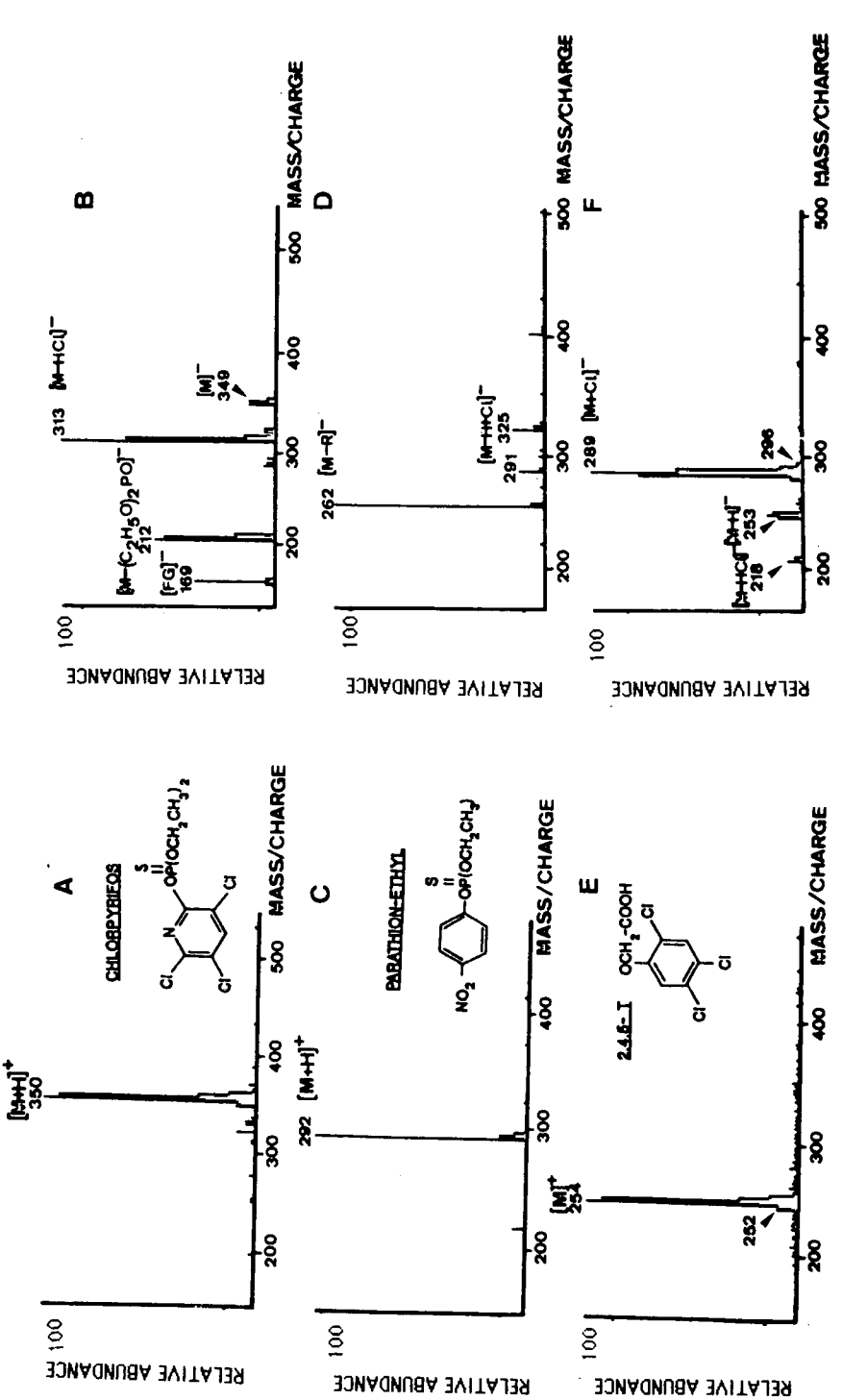

Fig. 6. Flow-injection PI (A, C, E) and NI (B, D, F) mode TSP mass spectra of chlorpyrifos (A, B), parathion-ethyl (C, D) and 2,4,5-T (E, F). Carrier stream for compounds A and B, cyclohexane, and for C–F, dichloromethane; flow-rate, 0.7 ml/min; amount injected, 500 ng; TSP vapour and ion source temperature, 200°C.

[36] and TSP-LC–MS [37] have also been reported. The method reported here involves the use of a post-column extraction system of difenzoquat with appropriate sulphonate counter ions. This is a sequel to the method developed by De Ruiter *et al.* [38] for the analysis of cationic detergents with sulphonate counter ions via post-column ion-pair formation and subsequent extraction. Several sulphonated counter-ions (methyl orange, dodecane sulphonate, Acid Blue 113 and Mordant-Red 9 [39]) were tested. All these sulphonates showed good extraction efficiencies (80–100%) with difenzoquat when it was extracted from an aqueous phase [acetonitrile–water (60:40) containing $1 \cdot 10^{-4}$ *M* of the sulphonate] to an organic phase [cyclohexane–dichloromethane–*n*-butanol (45:45:10)]. In Fig. 7 the TSP mass spectra obtained with flow injection of difenzoquat using a carrier stream of acetonitrile–water (A) and via the post-column extraction system, where difenzoquat is extracted as an ion pair with Acid Blue 113 (B), are shown. The limit of detection after the post-column extraction and under full scan conditions was 100 ng, which is comparable to that obtained by post-column reduction using alkaline sodium dithionite and UV detection [32,35]. The UV spectra of difenzoquat in the carrier stream and in the extraction solvent exhibited maxima at 254 nm, being slightly different on the blue side of the spectrum, thus indicating that UV spectra are dependent on the eluent composition [40]. Although the observed changes in both UV spectra could prove the formation of the ion pair (difenzoquat–sulphonate complex) and its extraction into the organic phase, nothing could be detected by TSP-MS.

The TSP mass spectra of difenzoquat in the PI mode showed a base peak at *m/z* 235 and a second abundant ion at *m/z* 249, corresponding to the cations [($C_{17}H_{17}N_2$)

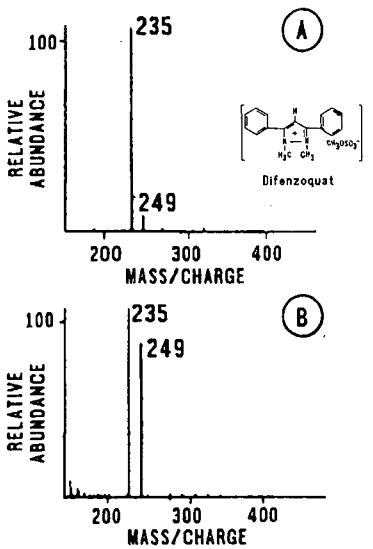

Fig. 7. TSP-mass spectra of difenzoquat obtained (A) using a reversed-phase eluent of acetonitrile–water and (B) after the post-column extraction system with cyclohexane–dichloromethane–*n*-butanol (45:45:10) at 1 ml/min; TSP vapour and ion source temperature, 200°C, with stem temperatures of (A) 100°C and (B) 86°C.

$-CH_3+H]^+$ and to $[(C_{17}H_{17}N_2)]^+$, respectively. The relative abundance of the latter was higher when the post-column ion-pair extraction system was employed (Fig. 7B). This can be explained by the lower vaporizer temperature used in the post-column set-up. The lower vaporizer temperature was needed for a stable signal, because the higher volatility of the extraction solvent is lower than that of water. A consequence of this lower vaporizer temperature is a decreased thermally induced dissociation. Hence the ion at *m/z* 249 had a higher abundance in the extraction set-up. The observed mass spectra match closely those obtained in fast atom bombardment (FAB)-MS experiments [36]. The background TSP spectra contained a peak at *m/z* 344, resulting from Acid Blue 113, and corresponding to a cleavage near the azo group. This fragment was also observed in FAB-MS experiments [39].

CONCLUSIONS

The features of different eluents for pesticide confirmation using both the PI and NI mode in TSP-LC–MS have been discussed. The influence of either the PI or NI mode on the sensitivity depends, in general, on the compound of interest. For chlorinated phenoxy acids, the NI mode is recommended using RP eluents whereas for carbamates and chlorotriazines the PI mode is preferred. For the other groups of pesticides the differences are not so pronounced and, consequently, both modes can be used. With different eluents significant differences are observed in the PI mode for the adduct ions, although the base peak is always the $[M+NH_4]^+$ ion. When the NI mode is used the base peak generally changes to the anion attachment peak of the eluent additive used. Different eluent additives can be applied in both the PI and NI modes to obtain more information for identification of the different groups of pesticides.

The use of NP eluents in TSP-LC–MS offers the advantage of improved detection in the PI mode for electronegative compounds such as chlorinated phenoxy acids. In the NI mode additional structural information is obtained and the fragment ions show a great resemblance to the ions observed under NCI-MS conditions.

The post-column ion-pair extraction system increases the selectivity and allows the introduction of polar herbicides in TSP-LC–MS. However, there is still a need to improve the analytical system for the analysis of quaternary ammonium compounds by LC–MS with ion-pair extraction, because the volatility of the ion-pairing reagent should be chosen carefully.

The possibilities of using different LC mobile phase compositions in both the PI and NI modes and their application to the identification and determination of pesticide residues at the ppb level in soil samples have been demonstrated.

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Interference removal in the organic trace-level analysis of aqueous environmental samples by on-line liquid chromatographic preconcentration techniques with two precolumns[a]

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ABSTRACT

On-line sample handling is used to enrich trace organic compounds in order to isolate and preconcentrate them prior to their liquid chromatographic separation. The main difficulty is that aqueous samples are complex matrices and therefore many interferences are also preconcentrated, giving rise to a high background. It is shown that matrix interferences are not a problem for determination at the ppb (μg/l) level in natural waters, but have to be removed to reach the 0.1 ppb level in drinking water (EEC requirement for many pesticides). Preconcentration is then optimized in terms of interference removal: two precolumns are coupled in series for the preconcentration step, the first acting as an interferent filter and the second trapping the analytes of interest. The methodology is different whether a specific sorbent for the analytes exists or not. Applications to the determination of moderately and rather polar herbicides (chlorotriazines and phenylureas) are presented. When no selective sorbent can be found, it is possible to optimize the fractionation between many rather apolar interferences and analytes by coupling two non-selective materials. Determination of polar herbicides is solved by coupling two precolumns, one packed with an alkylsilica (C_{18} or C_8) and the other with a styrene–divinylbenzene copolymer (PRP-1). The choice of the *n*-alkylsilica (carbon loading and chain length) is then important and is directly related to the solute polarity because the solutes must be slightly retained to have recoveries as high as possible on the PRP-1 precolumn. When a selective sorbent such as an ion exchanger can be selected, inorganic and organic interferences are efficiently removed by a two-step preconcentration and a first PRP-1 precolumn. Detection limits are therefore below 0.1 μg/l.

INTRODUCTION

In recent years, for the determination of trace amounts of organic pollutants in environmental aqueous samples, preconcentration techniques based on liquid–solid sorption extraction have grown in interest as an alternative to laborious and time-consuming liquid–liquid extraction [1–3]. Solid-phase extraction (SPE) is often described as an off-line sample preparation technique, involving the use of disposable

[a] In memory of R. W. Frei.

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prepacked cartridges with a subsequent gas chromatographic (GC), GC–mass spectrometric (MS) or liquid chromatographic (LC) analysis of the extract. Although the off-line procedure usually shortens the time of sample handling, a certain amount of tedious labour remains. On-line trace enrichment and analysis is obviously advantageous from the point of view of sensitivity, rapidity and possible automation. As there is no manual handling of the concentrated sample, the entire sample can be analysed quantitatively and no contamination risk occurs [4]. Although a few examples of on-line SPE preconcentration and GC analyses have been described [5–8], when looking for trace organics in environmental aqueous samples reversed-phase chromatography is the easiest method to be coupled on-line with SPE preconcentration: the residual water does not have to be removed before the analysis and LC offers much potential for the trace determination of many thermodegradable and/or non-volatile organics [9,10].

There is now a great need for methods suitable for determining concentrations below 0.1 μg/l (0.1 ppb) of trace constituents, which is the EEC maximum allowed concentration of a single pesticide in drinking water. Quantitative and accurate determinations at this level require detection limits of the analytical method at a lower level, *i.e.*, 10–50 ppt. When using a precolumn packed with a non-selective reversed-phase sorbent, many other components of the sample matrix will usually be co-extracted and co-eluted together with the analytes. This problem is not important if the concentration of analytes is much higher than that of the matrix components, but it has to be solved when analytes are to be determined at low ppb concentrations, as they are then masked by the complex pattern of interfering substances. Therefore, the detection limits can be strongly influenced by the number and concentration of co-eluting impurities in the sample matrix and an analytical method carried out with spiked LC-grade water samples at the 10–100 ppt level cannot be applied at this level with natural water samples owing to the high interference background. It is then necessary to minimize interferences to lower the detection limits.

On-line sample handling using precolumn technology is a powerful approach to obtaining selective preconcentrations, as several precolumns can be coupled in series to fractionate a complex mixture [11–13]. The sample volume can be increased to 500 ml and more, so that high enrichment factors can be obtained [14], making it possible to use simple UV detection. When concentrating organics from water samples, most of the widely used sorbents are non-selective reversed-phase materials such as *n*-alkylsilicas and to a lesser extent copolymer-based (PRP-1) and carbon-based sorbents [1,11–15]. More selective sorbents have been used, such as metal-loaded phases and ion exchangers [16–24].

The advantage of a selective sorbent is that part of the matrix components does not interfere. Nevertheless, for some relatively polar organic pollutants, no selective sorbent is available and interference removal is still a problem. The aim of this work was to optimize the use of precolumn technology in terms of interference removal: two precolumns are coupled in series for the preconcentration step, the first acting as an interference filter and the second trapping the analytes of interest with high recoveries. We discuss the sorbents to be packed in the two precolumns depending on the solute polarity and on the sample volume to be handled for the required UV or electrochemical detection. The methodology and results are different and depend on whether a selective sorbent for analytes can be found or not. Determination of chlo-

rotriazine and phenylurea herbicides is considered as an example of the two methods. When no selective packing can be used, two reversed-phase materials are coupled [*n*-alkylsilica and a styrene–divinylbenzene copolymer (PRP-1)]. In that case, there is an optimization of the complex mixture fractionation; the choice of the *n*-alkylsilica (carbon loading and chain length) is then important and is directly related to both the nature of the matrix components and the polarity of the solutes, because the solutes must be slightly retained to have high recoveries on the PRP-1 precolumn. Information about the polarity of matrix interferences in river and drinking water samples is also presented. A comparison of the coupling of a non-selective sorbent to remove interferences with a selective sorbent is made for chlorotriazine determinations.

EXPERIMENTAL

Apparatus

On-line percolation of water was performed using a Milton Roy pump (LDC, Riviera Beach, FL, U.S.A.). Precolumn elutions and analyses were carried out with a Varian (Palo Alto, CA, U.S.A.) Model 5500 liquid chromatograph equipped with a Model 200 variable-wavelength spectrophotometer and a Coulochem Model 5100 electrochemical detector (ESA, Bedford, MA, U.S.A.). Precolumn and analytical column switching was connected via two Rheodyne (Berkeley, CA, U.S.A.) valves. Quantitative measurements of peak areas were provided by a CR3A integrator–computer from Shimadzu (Kyoto, Japan).

Stationary phases and columns

The analytical columns were 15 cm × 4.6 mm I.D. stainless-steel columns prepacked with 5-μm octadecylsilica Nucleosil C_{18} (Macherey, Nagel & Co., Düren, Germany) or with 5-μm octadecylsilica Spherisorb ODS-2 (Whatman, Clifton, NJ, U.S.A.). Water samples were preconcentrated on 10 mm × 2.1 mm I.D. stainless-steel precolumns available from Chrompack (Middelburgh, The Netherlands), which were packed manually with a thick slurry using a microspatula or with a thin slurry using a syringe. The stationary phases packed in precolumns were 10-μm octylsilica RP-8, 10-μm octadecylsilica RP-18 (Merck, Darmstadt, Germany), 10-μm octadecylsilica Partisil-10 ODS, carbon loading 5% and Partisil-10 ODS-2, carbon loading 15%, from Whatman, the spherical 10-μm styrene–divinylbenzene copolymer PRP-1 (Hamilton, Reno, NV, U.S.A.) and the sulphonic acid-type resin-based cation exchanger BC-X8, 15–20 μm (Benson, Reno, NV, U.S.A.). A 15 mm × 3.2 mm I.D. precolumn prepacked with a 7-μm PRP polystyrene–divinylbenzene polymer (Brownlee Columns, Applied Biosystems, San Jose, CA, U.S.A.) was also used.

Chemicals

High-performance liquid chromatographic (HPLC)-grade acetonitrile was obtained from Rathburn (Walkerburn, U.K.) and methanol from Prolabo (Paris, France). LC-grade water was prepared by purifying demineralized water in a Milli-Q filtration system (Millipore, Bedford, MA, U.S.A.). Other chemicals were from Prolabo, Merck or Fluka (Buchs, Switzerland).

Stock solutions of selected solutes were prepared by weighing and dissolving them in methanol. LC-grade water samples were spiked with these solutions at the

ppb or ppt level. The final standard solutions did not contain more than 0.5% of methanol.

Procedure

The experimental set up is described in ref. 14. When using two non-selective precolumns, the water sample was introduced on the two precolumns in series; then the precolumns were flushed with 4 ml of 10^{-3} *M* perchloric acid. Each precolumn was separately coupled to the analytical column by switching a valve and back-flush eluted by an acetonitrile gradient via the HPLC pump. Precolumns in series were cleaned with pure acetonitrile and regenerated with 25 ml of 10^{-3} *M* perchloric acid.

When using a selective cation-exchange precolumn, the following procedure was adopted. A natural water sample adjusted to pH between 6 and 8 (if necessary) was percolated through the PRP-1 precolumn alone. After flushing with 2 ml of LC-grade water, the PRP-1 precolumn was coupled to the 10-mm long cation-exchange precolumn and 3 ml of a mixture containing 25% of acetonitrile and water acidified at pH 1 with perchloric acid were percolated through the two precolumns in series. After flushing the two precolumns with 2 ml of LC-grade water, the cation-exchange precolumn was coupled alone to the C_{18} analytical column and back-flush eluted by an acetonitrile gradient with lithium perchlorate-perchloric acid (0.05 *M*) at pH 4 via the HPLC pump. The PRP-1 precolumn was cleaned with 10 ml of pure acetonitrile and regenerated with 20 ml of LC-grade water. The cation-exchange precolumn was regenerated with 25 ml of 10^{-3} *M* perchloric acid.

Drinking water samples were analysed without any filtration. River water samples were filtered over a glass-fibre filter (Whatman GF/F).

RESULTS AND DISCUSSION

The selected herbicides for this study are listed in Table I and are representative of a range of moderately to relatively polar compounds, as shown by their water–octanol partition coefficients [25]. As an indication of polarity, the hydrophobic constants of simazine and atrazine are comparable to those of phenol (log P = 1.48) and 2-chlorophenol (log P = 2.16), respectively. For phenylurea herbicides, no selective sorbent has been reported; for some of them, the detection limit can be slightly improved by the use of electrochemical detection or by a derivatization reaction [26].

TABLE I

WATER–OCTANOL PARTITION COEFFICIENTS (LOG P_{oct}) AND IONIZATION CONSTANTS (pK_a) FOR SELECTED HERBICIDES

Phenylureas		Log P_{oct}	Triazines		Log P_{oct}	pK_a
Abbreviation	Name		Abbreviation	Name		
M	Metoxuron	1.98	S	Simazine	1.51	1.65
C	Chlortoluron	2.55	A	Atrazine	2.05	1.68
I	Isoproturon	2.65	P	Propazine	2.59	1.85
			T	Terbutylazine	2.65	1.95

Chlorotriazines can be considered as ionizable compounds (with low ionization constants) and can be selectively concentrated by a cation-exchanger [27].

A simple calculation indicates the sample volume to be handled in order to reach the required detectable amount: the average UV or electrochemical detection limit of analytes being 1–5 ng [28], a 100–500-ml sample volume is necessary to reach the 10 ppt level for spiked LC-grade water samples and provide easy determinations at the low ppb level in river water samples. Therefore, the analyte retention in water with the selected sorbent must be high, as the precolumn dimensions are small in order to avoid band broadening of analytes during their transfer from the precolumn to the analytical column (for a classical 15 cm × 0.46 cm I.D. column, the precolumn dimensions should not exceed 1 cm × 0.46 cm I.D.) [29]. Among the non-selective sorbents, previous studies have shown that the styrene–divinylbenzene copolymer-based PRP-1 sorbent provides efficient retention of relatively polar aromatic compounds in aqueous samples [14].

Interference removal using two non-selective materials

Description of the methodology. When using two non-selective reversed-phase materials for the preconcentration, the fractionation has to be optimized to remove many interferences on the first precolumn and concentrate analytes on the second. Retention of analytes depends on the sample volume, on the sorbent characteristics and on the nature of the analyte. Sorption can be appreciated by determining breakthrough volumes on each precolumn. Breakthrough volumes of apolar solutes on the first alkylsilica precolumn are high and these solutes are recovered only on the first precolumn; polar solutes, not retained on the alkylsilica, are recovered only on the second precolumn, but many moderately polar compound are revovered on both precolumns and the respectively preconcentrated amounts depend on the sample volume and on the analyte breakthrough volumes on each precolumn. Quantitative determinations are still possible and a direct experimental method of recovery measurement has been described [14]. It consists in measuring the peak areas obtained with a 10-ml preconcentration of spiked LC-grade water. The 10-ml volume is chosen so that no breakthrough occurs from the alkylsilica precolumn. Then the sample volume is increased but the solute concentration is decreased to have a constant amount in each sample. For each percolation, the peak areas are measured on the chromatograms corresponding to the elution of each precolumn. Recoveries are calculated by dividing these values by the peak-area values obtained for the first 10-ml percolation. This experimental measurement of recovery has the advantage of taking into account the transfer and the desorption processes from the column to the analytical column. Breakthrough of solute on the alkylsilica precolumn starts when its recovery decreases on this precolumn. If no breakthrough occurs from the PRP-1 precolumn, the sum of recoveries from alkylsilica and PRP-1 is 100%, and each recovery represents the part of the analyte preconcentrated on each precolumn. Breakthrough on the PRP-1 precolumn starts when the sum of the recoveries begins to fall below 100%. For the selected analytes, breakthrough volumes higher than 500 ml have been measured with a 15 mm × 3 mm I.D. precolumn packed with the PRP-1 sorbent.

Effect of n-alkylsilica carbon loading. Four *n*-alkylsilica sorbents, one C_8 and three C_{18} having different carbon loadings, were tested to investigate their potential for interference removal with drinking and river water samples.

Large volumes of raw drinking water and river water were spiked with 1 μg/l of each analyte. Four replicate runs were performed with 500-ml samples of each type of spiked water which were passed through the two precolumns in series, the first packed with one of the tested alkylsilicas and changed between two runs and the second packed with the PRP-1 sorbent and unchanged. In each run, the two precolumns were eluted on-line with UV and electrochemical detection. Fig. 1 shows the UV chroma-

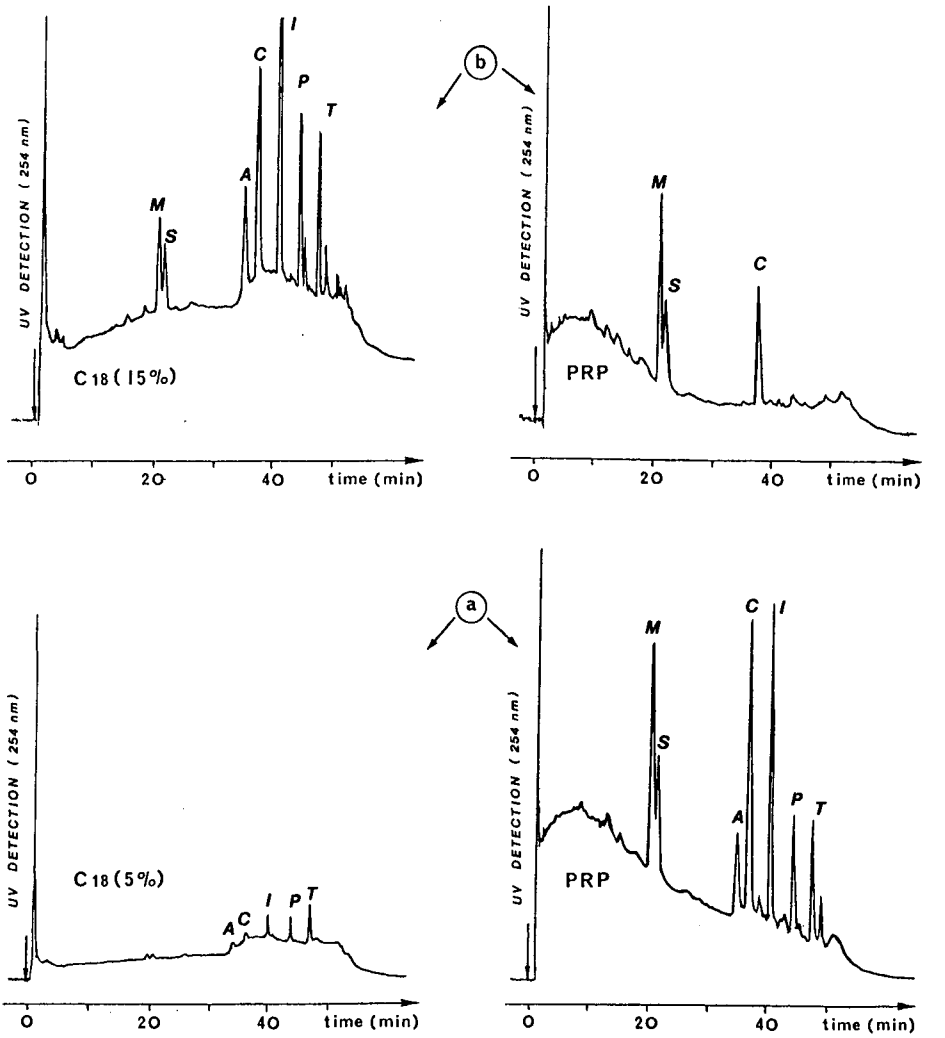

Fig. 1. On-line preconcentration of 500-ml drinking water samples spiked with 1 μg/l of each herbicide. See Table I for peak identification. Preconcentration through two precolumns in series packed with (a) octadecylsilica Partisil-10 ODS with 5% carbon loading and PRP-1 and (b) octadecylsilica Partisil-10 ODS-2 with 15% carbon loading and PRP-1 at a flow-rate of 5 ml/min; elution to the analytical column (15 cm × 4.6 mm I.D.) packed with 5-μm Spherisorb ODS 2 silica at a flow-rate of 1.5 ml/min. Mobile phase: acetonitrile gradient with a solution of potassium acetate–acetic acid (0.1 *M*) at pH 4.6; gradient from 14.5% to 23.5% of acetonitrile from time 0 to 40 min, to 37% at 60 min and to 100% at 80 min. UV detection at 254 nm; sensitivity, 0.016 a.u.f.s.

tograms obtained for the elution of both precolumns when concentrating a 500-ml drinking water sample and when using a C_{18} silica with a low carbon content (Fig. 1a) and a high carbon content (Fig. 1b). First, it can be observed that the ratio of the amount preconcentrated by both precolumns depends greatly on the carbon loading of the first precolumn: the 5% carbon silica slightly retains the more apolar analytes and almost everything else is recovered on the PRP-1 precolumn. In contrast, with the 15% carbon silica, analytes are mainly retained on the first precolumn, and only the more polar ones are recovered from the second PRP-1 precolumn. Fig. 1 shows also that when analytes are to be determined at the 1 μg/l level in drinking water samples,

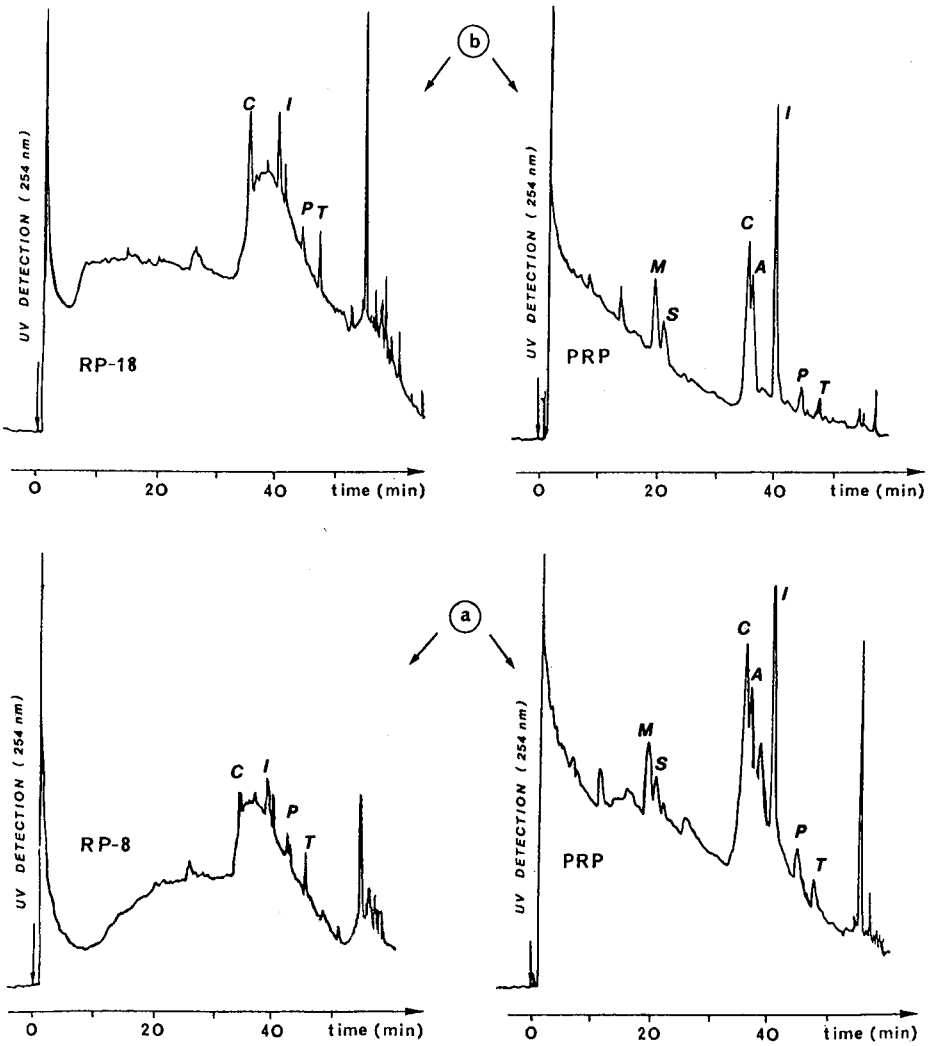

Fig. 2. On-line preconcentration of 500-ml River Yerres samples (March 89) spiked with 1 μg/l of each herbicide. Preconcentration through two precolumns in series packed with (a) octylsilica RP-8 and PRP-1 and (b) octadecylsilica RP-18 and PRP-1 at a flow-rate of 5 ml/min. UV detection at 254 nm; sensitivity, 0.032 a.u.f.s.; other conditions as in Fig. 1.

the problem of matrix interferences is not important and a single precolumn packed with a high carbon content C_{18} silica or a PRP-1 sorbent can be used. If analytes are to be determined at the 0.1 μg/l level, one has to divide each peak height by 10 and it is clear that peaks will not then emerge greatly from the interference background.

Information about the polarity of interferences in this drinking water sample is also obtained. The 5% C_{18} silica can retain very apolar solutes which would be eluted at the end of the gradient (above 50 min), so that the matrix does not contain many very apolar components, having UV absorption properties at 254 nm; the PRP-1 chromatogram in Fig. 1a indicates a larger amount of interferences during the first 30 min, which correspond to rather polar and moderately polar components. These observations are confirmed by the chromatograms in Fig. 1b: the 15% C_{18} silica traps many moderately polar interferences and only the more polar ones are recovered on the PRP-1 precolumn, as shown during the first 20 min of the gradient.

Interferences are more numerous in river water, as can be seen in Fig. 2, which shows the chromatograms obtained with similar experiments to those in Fig. 1 when using RP-8 (Fig. 2a) and RP-18 (Fig. 2b) in the first precolumn for the preconcentration of a 500-ml river water sample spiked with 1 ppb of each analyte. In comparison with Fig. 1, the background is much more important. As expected, the matrix components trapped by RP-18 are more numerous than those trapped by RP-8, but the baseline obtained on the PRP-1 chromatogram is very different, owing to the "interference filter effect" of the first precolumn. For instance, if the analytes of interest are atrazine or chlortoluron, their determination will be more accurate when using RP-18 as the first precolumn because with RP-8 there are too many interferences left in this area of the chromatogram. When using the 15% carbon content C_{18} silica (not shown), the filter effect is higher but the compounds are trapped too much on the first precolumn with the interfering material. Table II reports the recoveries obtained for the four couplings studied above on the PRP-1 precolumn from 500-ml samples. With RP-18 as the first precolumn, the recoveries of moderately polar compounds are between 80 and 85% and those of apolar compounds between 60 and 40%. As the detection limit depends on the baseline quality, there is a compromise between efficiently minimizing interferences and obtaining good recoveries of analytes on the second precolumn. This compromise depends on the polarity of both analytes and matrix components. For many types of drinking water and river water, coupling

TABLE II

RECOVERIES OF HERBICIDES ON THE PRP-1 PRECOLUMN WHEN IT IS COUPLED TO A FIRST PRECOLUMN PACKED WITH DIFFERENT *n*-ALKYL-BONDED SILICAS

Mean values obtained from triplicate measurements from 500-ml samples spiked with 1 μg/l of each analyte. See Table I for herbicide identification.

n-Alkylsilica	Recovery on PRP-1 precolumn (%)						
	M	C	I	S	A	P	T
ODS 5%	98	98	94	97	93	87	78
ODS-2 15%	70	37	13	60	18	12	10
RP-8	97	91	86	98	87	72	59
RP-18	98	85	80	97	80	60	40

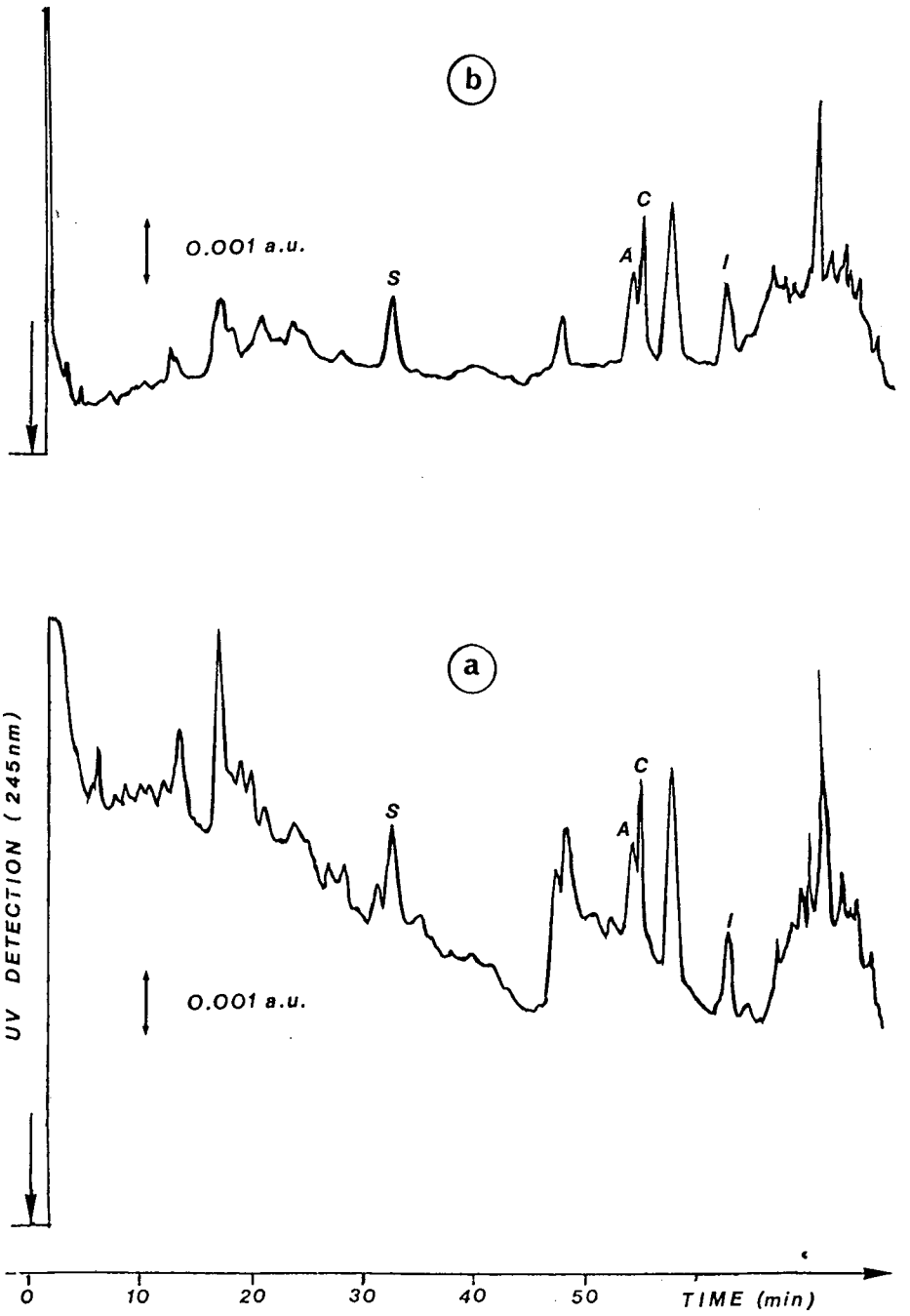

Fig. 3. Clean-up effect. On-line analysis after preconcentration of a non-spiked 500-ml River Seine sample (Paris, April 24th, 1989); elution of the PRP-1 precolumn (a) without clean-up and (b) with flushing with 1 ml of water modified with 10% of acetonitrile. UV detection at 245 nm; other conditions as in Fig. 2 except the analytical column was packed with Nucleosil C_{18}.

RP-18 and PRP-1 has been shown to be a suitable choice for a good fractionation between interferences and moderately polar analytes.

Further clean-up. It is possible to flush the PRP-1 precolumn before its elution by a small volume of water modified with an organic solvent in order to remove some more interferences. This method is widely applied to on-line and off-line preconcentration of apolar analytes which are not eluted from the precolumn by the flushing solution. However, for more polar analytes, elution can occur and tests should be carried out to verify that there is no loss of sorbed analytes during this flushing.

Experiments were carried out with river water samples. Volumes of 500 ml of spiked river water samples were preconcentrated through the two precolumns and the PRP-1 precolumn was flushed before its on-line elution with a few millilitres of water modified with 10–20% of acetonitrile (ACN). With 3 ml of water containing 20% of ACN, some analytes are eluted. Decreasing the ACN content to 10% still does not prevent the loss of the more polar compounds. With 1 ml containing 10% of ACN, the recoveries of metoxuron and simazine are 80% and of other analytes 100%. One can expect this flushing to have a small effect only on the more polar interferences, but Fig. 3 shows that this flushing effect helps to obtain a clearer chromatogram: the background is much higher without flushing (Fig. 3a), especially at the beginning of the chromatogram. In this river water sample, four herbicides have been identified: simazine (*ca.* 0.6 ppb), atrazine (*ca.* 0.6 ppb), chlortoluron (*ca.* 0.2 ppb) and isoproturon (*ca.* 0.2 ppb).

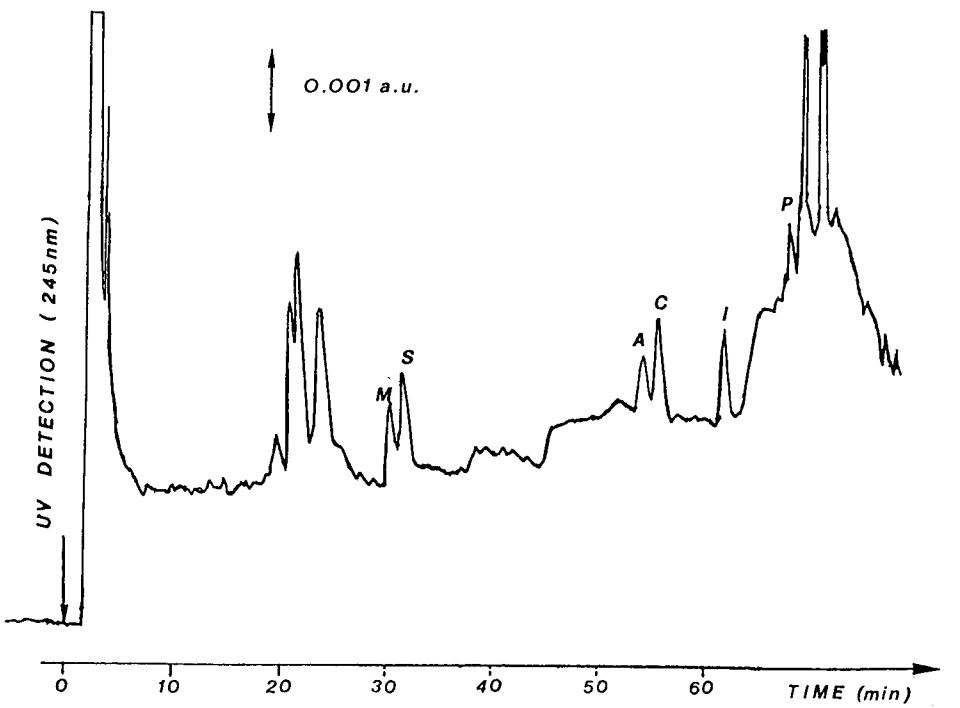

Fig. 4. On-line analysis of the PRP-1 precolumn after preconcentration of a 500-ml drinking water sample (Paris, May 1990) spiked with 0.1 μg/l of each herbicide. The PRP-1 precolumn was flushed with 1 ml of water modified with 10% of acetonitrile. UV detection at 245 nm; other conditions as in Fig. 3.

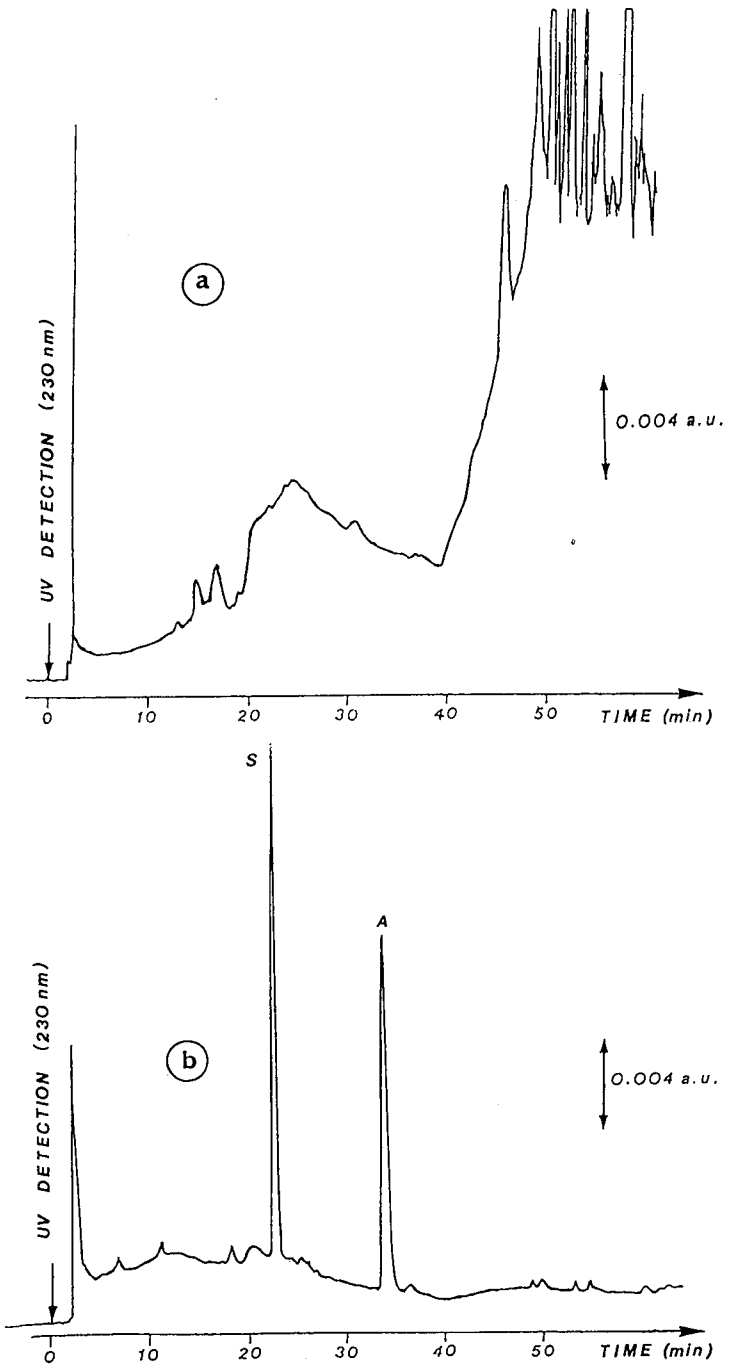

Fig. 5. Filter effect of the PRP-1 precolumn. On-line analysis after preconcentration of a non-spiked 500-ml River Seine sample (Paris, April 24th, 1989; same sample as in Fig. 3). (a) On-line analysis of the PRP-1 precolumn; (b) on-line analysis of the cation-exchange precolumn; preconcentration at pH 6 through a 15 mm × 3.2 mm I.D. precolumn packed with 7-μm copolymer PRP-1, transfer to 10 mm × 2 mm I.D. precolumn packed with 15–20-μm BC-X8 cation exchanger with 3 ml of 0.1 *M* perchloric acid modified with 25% of acetonitrile. Analytical column: 15 cm × 4.6 mm I.D. packed with 5-μm Nucleosil C_{18}; mobile phase, acetonitrile gradient with a 0.05 *M* perchloric acid–lithium perchlorate at pH 4 at a flow-rate of 1.5 ml/min; gradient, 15% acetonitrile from 0 to 20 min, 23.5% from 20 to 35 min and up to 46% at 60 min; UV detection at 230 nm.

Detection limits. In river water samples, under the selected chromatographic conditions, *i.e.*, fractionation for the preconcentration and further clean-up, the detection limits (signal-to-noise ratio ≈ 3) in 500-ml samples are about 0.1 ppb for triazines with UV detection at 254 nm and 0.05–0.1 μl/l for phenylureas with either UV detection at 245 nm or electrochemical detection.

The detection limits obtained in 500-ml drinking water samples by the same method are lower than those obtained in river water, owing to the less numerous interferences. In Fig. 4, the PRP-1 chromatogram of a 500-ml drinking water sample spiked with 0.1 μg/l of each analyte indicates that quantitative analyses can be performed at this level.

Interference removal using a non-selective and a selective sorbent (chlorotriazines)

Description of the methodology. Chlorotriazines can be selectively preconcentrated by a cation exchanger if the pH of the aqueous sample is adjusted below 1, which involves the use of a polymer-based cation exchanger. Nevertheless, the sample cannot be directly percolated through a precolumn packed with this specific sorbent because of the large amount of inorganic cations which would quickly overload the precolumn. A sample clean-up can remove these inorganic cations (precipitation with sodium oxalate and complexation with EDTA), but traces of inorganics are still present and the sample volume of raw water which can be passed through the cation-exchange precolumn is limited to 30 ml [24]. To avoid the direct percolation through the cation exchanger and to remove both organic and inorganic interferences, a two-step preconcentration is carried out, as described in detail previously [27]. It is based on the difference in retention observed on a PRP-1 sorbent for triazines, depending on whether they are in their neutral or cationic form. The first step consists in percolation of the 500-ml sample adjusted to pH 7 through a PRP-1 precolumn. Then a small volume of well demineralized water at pH 1 and modified with 20% of ACN allows the organic cations to be desorbed alone from this PRP-1 precolumn and to be transferred and preconcentrated by a second precolumn packed with a cation exchanger which is analysed on-line. Fig. 5 shows the analysis of the same 500-ml river water sample as in Fig. 3. Only simazine and atrazine are detected of the four herbicides present, but, in comparison with Fig. 3, the peaks are much more intense and the baseline is free from interferences (Fig. 5b). This is due to the efficient removal of many neutral interferences which are left on the PRP-1 precolumn, as visible in the eluate of the PRP-1 content in Fig. 5a. Consequently, it was possible to detect triazines at 230 nm, close to their maximum UV absorbance (220 nm). Detection at 230 nm was impossible in Fig. 3 owing to a more important background, indicating a higher UV absorbance of interferences at 230 nm. The inverse was observed in the eluate of the cation exchanger. Fig. 6 shows the UV chromatograms, at two different wavelengths, corresponding to the same cation exchange eluate from a 500-ml river water sample; interferences showed a higher UV absorbance at 254 nm than that obtained at 230 nm, thus indicating the presence of protonated aromatic compounds. The corresponding electrochemical detection was free from interferences, so that these aromatic interferences were not oxidizable.

Detection limits. With this methodology coupling a powerful interference removal and a selective trapping of chlorotriazines, one can expect lower detection limits. In fact, the detection limits obtained with natural water samples are of the

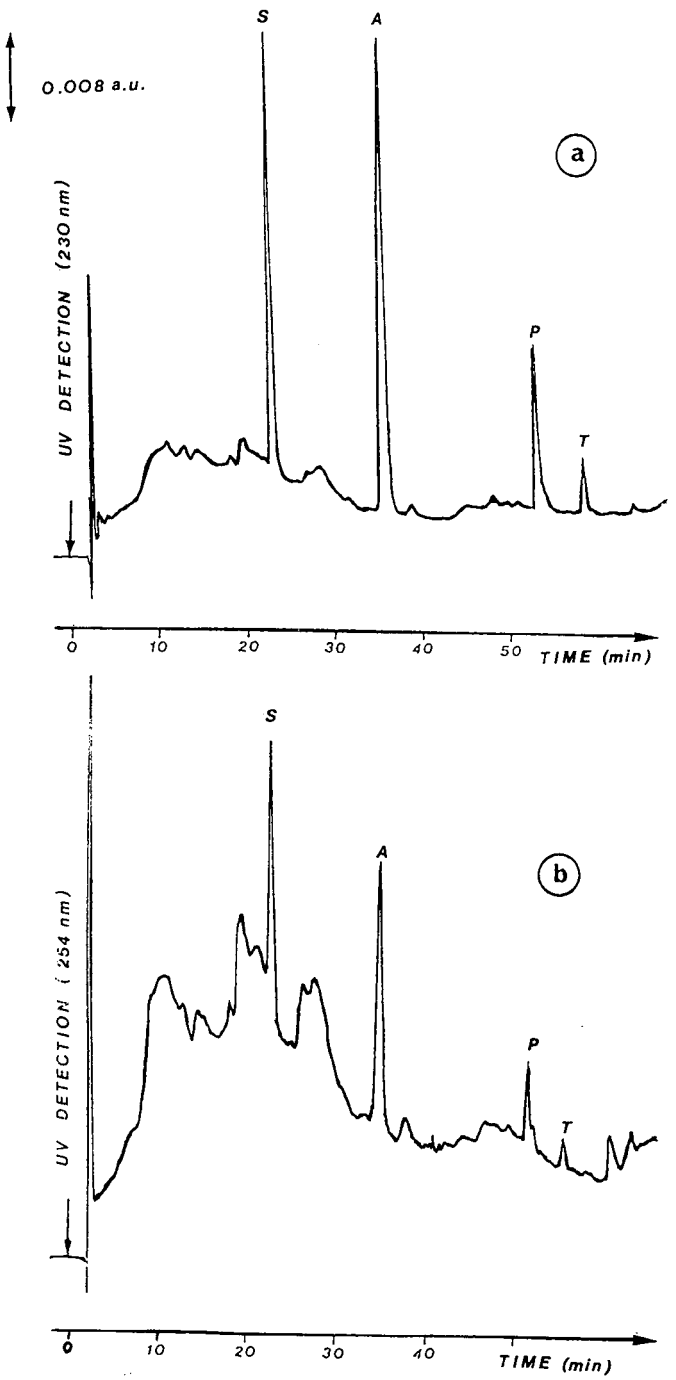

Fig. 6. Influence of the UV detection wavelength. On-line analysis of the cation-exchange precolumn after preconcentration of a 500-ml River Yerres sample spiked with 1 μg/l of each chlorotriazine. UV detection at (a) 230 nm and (b) 254 nm; experimental conditions as in Fig. 5.

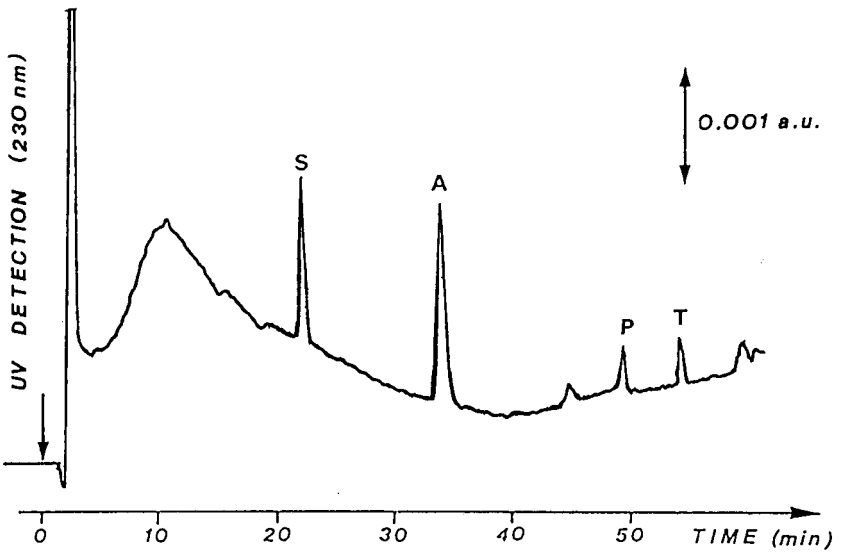

Fig. 7. Preconcentration and on-line analysis of a 500-ml drinking water sample from Paris, April 1990, containing simazine (30 ng/l), atrazine (35 ng/l) and propazine (20 ng/l), terbutylazine (20 ng/l). Experimental conditions as in Fig. 5.

same order as those obtained when injecting analytes directly into the analytical column. Fig. 7 shows the analysis of a drinking water sample containing 30 ng/l of simazine, 35 ng/l of atrazine and 20 ng/l each of propazine and terbutylazine. The detection limits are very low and have been calculated as 2–5 ppt in 500-ml samples (signal-to-noise ratio = 3). Baselines obtained with river water have the same quality as those obtained with drinking water samples, so that detection limits in river water samples are as low as those for drinking water samples. This clearly demonstrates that to lower detection limits, one has to search for selective preconcentrations. However, depending on the selective sorbent, it may be necessary also to remove interferences.

CONCLUSION

The results clearly show that there is an advantage in combining two precolumns for trace organic preconcentration from aqueous environmental samples with an efficient removal of many matrix interferences by the first precolumn and a selective trapping of analytes by the second. This permits analytes to be determined quantitatively and accurately below the 0.1 ppb level. When no selective sorbent can be found, an efficient fractionation of interferences from analytes can help to achieve low detection limits, which are higher than those obtained with the use of a selective sorbent.

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CHROMSYMP. 2314

Sensitive liquid chromatographic determination of alkyl-, nitro- and chlorophenols by precolumn derivatization with dansyl chloride, postcolumn photolysis and peroxyoxalate chemiluminescence detection

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ABSTRACT

A liquid chromatographic method is described for the determination of alkyl-, nitro- and chlorophenols at sub-ppb levels using a very sensitive and selective detection system. The phenols are labelled by two-phase dansylation. The deprotonated phenolic anions are extracted as tetrabutylammonium ion pairs into an organic phase in which dansyl chloride is dissolved. After derivatization, the excess of reagent is removed on an amino-bonded column; dansyl chloride reacts with the amino groups whereas the phenol derivatives are not retained. Chromatography is carried out with a methanol–water gradient followed by photolysis of the derivatives. The strongly quenching electronegative nitro- and chlorophenol groups are photochemically removed from the derivative and the products, dansyl hydroxide and dansyl methoxide, are sensitively detected by peroxyoxalate chemiluminescence. Chemical excitation is carried out by adding 2-nitrophenyl oxalate and hydrogen peroxide dissolved in acetonitrile to the column eluate. Detection limits of about 0.01–0.1 ng/ml have been achieved and the method has been applied to the determination of several phenolic compounds in surface water.

INTRODUCTION

Nowadays several substituted phenolic compounds with low (pentachlorophenol) to relatively high (phenol and nitrophenols) polarity have to be monitored at sub-ppb levels of environmental samples such as surface water [1]. Established methods, including liquid–liquid extraction and/or distillation followed by liquid chromatography (LC) with UV detection [2–4], lack both sensitivity and selectivity. Recently, other methods, including electrochemical detection [5], mass spectrometry [6], gas chromatography [7], micellar electrokinetic chromatography [8] and capillary zone electrophoresis [9], have been described. However, as electrochemical detection is still not widely accepted for routine analysis, mass spectrometry is expensive, gas chroma-

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tography is less suitable for polar analytes in aqueous samples and electrokinetic methods lack concentration sensitivity, LC methods are to be preferred.

A fast and simple derivatization reaction for phenolic compounds was developed by De Ruiter *et al.* [10]. The labelling procedure involves ion-pair extraction of the deprotonated phenol with a tetrabutylammonium counter ion to an organic phase in which the non-polar derivatization reagent (dansyl chloride) is dissolved. The sensitivity can be significantly improved by application of a postcolumn photochemical reaction [11]. The highly fluorescent dansyl hydroxide and/or methoxide is/are liberated from the strongly quenching electronegative chlorophenol moiety. Detection limits of *ca.* 200 pg for tri, tetra- and pentachlorophenols were obtained. For the determination of the complete range of phenols at the required level, a 100-fold preconcentration would be necessary. In that event the determination of the polar phenols will cause problems because their breakthrough volumes on hydrophobic precolumns often are too small for such a preconcentration step [12].

Peroxyoxalate chemiluminescence (CL) detection of dansyl derivatives has been shown to be one to two orders of magnitude more sensitive than fluorescence detection [13–15]. The aim of this study was to combine the selective two-phase dansylation and photolysis of substituted phenols with sensitive peroxyoxalate CL detection. Further, the applicability of the method for a wide range of phenols, including polychloro-, alkyl- and nitrophenols, was investigated for tap water and river water samples.

EXPERIMENTAL

Chemicals

High-performance liquid chromatographic (HPLC)-grade solvents and amino-bonded (type 7088-03) and C_{18}-bonded (type 7020-03) SPE columns were purchased from Baker (Deventer, The Netherlands). Bis(2-nitrophenyl) oxalate (2-NPO) was synthesized as described [16]. 5-Dimethylamino-1-sulphonyl chloride (dansyl chloride), 2,4-dichlorophenol (DCP) and tetrabutylammonium bromide (TBABr) were purchased from Aldrich (Brussels, Belgium), phenol (P) from Baker, 4-nitrophenol (4-NP) from Fluka (Buchs, Switzerland), 2-nitrophenol (2-NP) and 2,4,6-trichlorophenol (TCP) from Janssen (Beerse, Belgium) and 2-chlorophenol (2-CP), pentachlorophenol (PCP), 2,3-, 2,5-, 3,4- and 3,5-dimethylphenol and 2-isopropyl-5-methylphenol from Merck (Darmstadt, Germany). 2,4-Dimethylphenol (DMP), 3-methyl-4-chlorophenol (CMP), 2,4-dinitrophenol (DNP), 2,4-dinitro-6-methylphenol, 2,4-dinitro-6-*sec.*-butylphenol (dinoseb) and 2,4-dinitro-6-*tert.*-butylphenol (dinoterb) were received as a gift from H. van der Zouwen (Provinciaal Waterleidingbedrijf Overijssel, The Netherlands). Stock solutions of all phenols were prepared in methanol and kept at 4°C in the dark. All other chemicals were of analytical-reagent grade.

Column liquid chromatography

The LC mobile phase flow-rate of 0.5 ml/min was delivered by a Gilson (Villiers-le-Bel, France) gradient system consisting of a Model 305 pump (A), a Model 302 pump (B), a Model 805 manometric module and a Model 811B dynamic mixer.

Pump A delivered methanol–100 m*M* imidazole buffer (pH 7.0) (97.5:2.5, v/v) and pump B methanol–2.5 m*M* imidazole buffer (pH 7.0) (2.5:97.5, v/v). The gradient programme was as follows: 75% A from 0 to 9.5 min, 85% A from 10 to 14.5 min, 95% A from 15 to 19.5 min, 100% A from 20 to 40 min and 100% to 75% A from 40 to 41 min. For every new run the column was allowed to equilibrate for at least 20 min at 75% A. A Rheodyne six-port valve with a 100-μl loop was used for introduction of samples onto a 200 × 3.1 mm I.D. column packed with 3-μm LiChrosorb RP-18 (Merck) by a standard slurry technique.

Detection system

After chromatography, the dansyl derivatives were irradiated in a photochemical reactor designed by Scholten *et al.* [17]. It was equipped with a Philips (Eindhoven, Netherlands) Model 93110E mercury lamp (90 W, 0.9 A). The reaction coil was a 130 × 0.3 mm I.D. × 1/16 in. O.D. polytetrafluorethylene (PTFE) capillary (helix diameter 72 mm), which was placed concentrically around the lamp. The reactor was cooled by a fan. Hydrogen peroxide and 2-NPO dissolved in acetonitrile at final concentrations of 50 and 5 m*M*, respectively, were mixed just before use and added to the column effluent (after photolysis) with a pulseless Isco (Lincoln, NE, USA) μLC-500 syringe pump. The mobile phase (0.5 ml/min) and the reagent stream (0.3 ml/min) were mixed with a standard Valco T-piece immediately before the detector. An ATTO (Tokyo, Japan) AC 2220 CL detector (operated at 700 V) equipped with a 65-μl spiral flow cell and a 470-nm cut-off filter was used for detection. Fluorescence detection was carried out with a Perkin-Elmer (Beaconsfield, U.K.) LS-2 fluorescence detector (λ_{exc} = 340 nm, λ_{em} = 470 nm).

Derivatization of phenolic compounds with dansyl chloride

Determination of phenolic compounds in the concentration range 10–1000 ng/ml. To 500 μl of an aqueous phenol-containing solution, adjusted to pH 12 with 1 *M* sodium hydroxide solution, 100 μl of an aqueous solution of TBABr (30 mg/ml, pH 12) and 600 μl of a solution of 0.1 mg/ml dansyl chloride in dichloromethane were added in a standard reagent tube (100 × 9 mm I.D.). The tube was capped with aluminium foil to avoid evaporation of dichloromethane and vortex mixed vigorously for 2 min. A 500-μl volume of the organic phase was slowly brought onto an amino-bonded SPE column (previously washed with dichloromethane and dried with nitrogen or air) and for 10 min the excess of dansyl chloride was allowed to react with the amino groups. The dansyl derivatives were eluted from the SPE column with 3 ml of dichloromethane, gently evaporated to dryness and the residue was dissolved in 500 μl of methanol–water (50:50, v/v). Finally, 100 μl were injected onto the reversed-phase column.

Determination of 2- and 4-nitrophenol, phenol and 2-chlorophenol in water samples at 0.1–10 ng/ml levels. A 2.0-ml volume of an aqueous phenol-containing solution, 300 μl of the TBABr solution and 800 μl of the dansyl chloride solution were treated as described above. A 500-μl volume of the organic phase was brought on to the amino-bonded SPE column and the same procedure was followed.

Determination of 2,4-dimethyl-, 3-methyl-4-chloro, 2,4-dichloro-, 2,4,6-trichloro- and pentachlorophenol in water samples at 0.1–10 ng/ml levels. A 3.0-ml volume of an aqueous acidified (pH 3.0) phenol-containing solution was preconcentrated on a

C_{18}-bonded SPE precolumn, desorbed with 3 ml of dichloromethane and evaporated to dryness under a stream of nitrogen. To the residue 100 μl of the TBABr solution, 500 μl of water (pH 12) and 600 μl of the dansyl chloride solution were added. The above procedure was then followed.

Analysis of water samples

Amsterdam tap water spiked with a number of selected phenols was either adjusted to pH 12 with 1 *M* sodium hydroxide solution for direct derivatization of the phenols or adjusted to pH 3 with 1 *M* nitric acid for preconcentration on a C_{18} precolumn. River water (Rhine) was filtered through a Millipore (Etten-Leur, Netherlands) 2-μm membrane filter prior to preconcentration and/or derivatization.

RESULTS AND DISCUSSION

Derivatization

Removal of excess of reagent. De Ruiter *et al.* [10] developed a rapid method for the dansylation of phenolic steroids using a two-phase system and phase-transfer catalysis. The deprotonated phenols are extracted as an ion pair with a tetrabutylammonium salt into an organic phase in which the apolar reagent (dansyl chloride) is dissolved. The reaction between the phenolic anion and dansyl chloride is very fast (*ca.* min at room temperature). The only disadvantage of the two-phase derivatization is that the excess of reagent remains in the same layer as the derivatized phenol. In normal-phase LC with fluorescence detection this is seldom a problem as dansyl chloride does not fluoresce. In reversed-phase LC, however, the excess of reagent will present a real problem as it will be partly converted into strongly fluorescent products.

Under the experimental conditions used in this study, the reagent appeared as a broad band with a retention close to that of the dansyl derivative of 2-CP. It was therefore necessary to remove the excess of reagent before injection into the LC system. In the literature the excess of reagent is often converted into a polar derivative by adding a small amount of a concentrated solution of a polar amine, *e.g.*, taurine [18] or glycine [19], after the derivatization of the analytes. This approach did not work here because a polar amine (or phenol) does not dissolve in the organic layer in which dansyl chloride is dissolved. Slightly more apolar amines such as ethylamine do remove the excess of reagent, but cause a broad tailing band in the early part of the chromatogram which interferes with the early-eluting dansylated phenol derivatives. Moreover, a large reagent band eluting from the column at t_0 causes a photomultiplier overload. This is especially inconvenient when using the ATTO AC-2220 CL detector, which has no overload safety.

De Ruiter *et al.* [11] described the used of amino-bonded TLC plates for the preparative dansylation of chlorophenols. The reagent dansyl chloride reacts with the amino groups and remains on the TLC plate with an R_F value of zero. This approach was slightly modified here by using amino-bonded SPE columns instead of TLC plates. After the normal two-phase dansylation procedure, 500 μl of the organic layer are brought onto a dry amino column where the excess of dansyl chloride will react with the amino groups. The volume should not exceed 500 μl in order to prevent breakthrough. The reaction time of the unreacted dansyl chloride with the amino

groups on the column should be at least 10 min at room temperature before elution of the dansyl derivatives from the amino column is carried out. Elution is typically performed with 3 ml of dichloromethane; the recovery of all dansylated phenols was 98–100%. For trace determinations (0.1–1 ng/ml) each analysis should be carried out with a new amino SPE column previously washed with dichloromethane and dried with nitrogen or air. The amino columns should preferably be dry because unreacted dansyl chloride is sometimes washed from a wet column. For analysis at higher levels (10–1000 ng/ml) it is possible to use the amino columns two or three times with intermediate washing with dichloromethane and drying. The chromatograms resulting after the removal of the excess of dansyl chloride were sufficiently clean even for the detection of dansyl derivatives of relatively polar phenols such as P and 2-CP. Injections of 100 μl were possible without excessive band broadening by using methanol–water (50:50, v/v) for injection. An example of a chromatogram with nine phenols derivatized according to the procedure described above is shown in Fig. 1.

Influence of the pH in the two-phase reaction. Using an aqueous phase with a pH of 10–11, irreproducible results were obtained for P and 2,4-DMP. Changing the pH

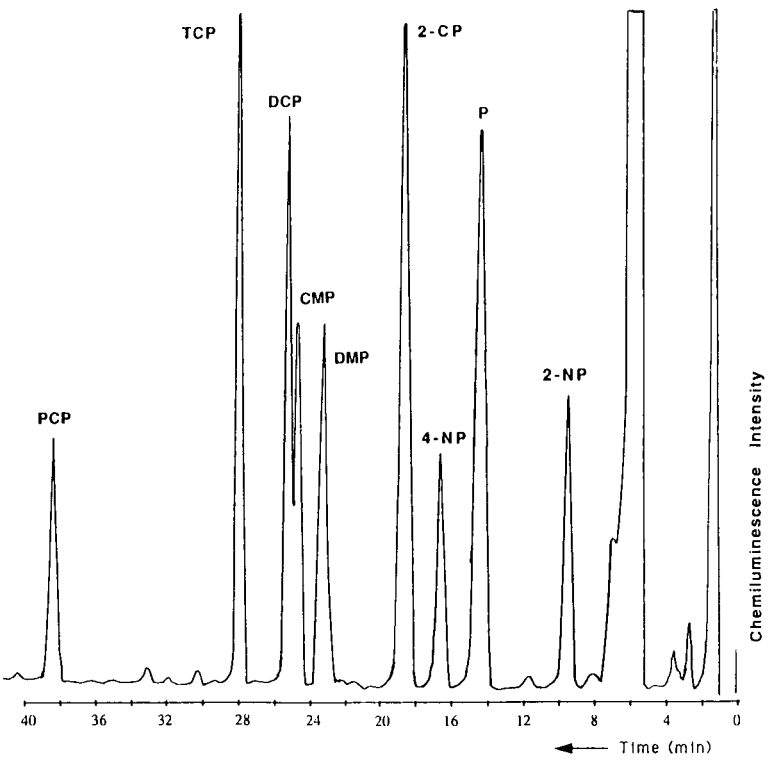

Fig. 1. Gradient LC with CL detection of nine dansylated phenols; 1.0–1.5 ng (phenol and chlorophenols) and 5.0 ng (mononitrophenols) injected. Sensitivity, 700 V; attenuation, 50. 2-NP = 2-nitrophenol; P = phenol; 4-NP = 4-nitrophenol; 2-CP = 2-chlorophenol; DMP = 2,4-dimethylphenol; CMP = 3-methyl-4-chlorophenol; DCP = 2,4-dichlorophenol; TCP = 2,4,6-trichlorophenol; PCP = pentachlorophenol. For derivatization and gradient elution programme, see Experimental.

to 12 improved the results for both phenols. Apparently, the pH of the aqueous phase should be at least one unit above the pK_a values of the phenolic compounds under investigation. Unfortunately, the higher chlorinated phenols containing three or more chlorine atoms are very unstable in pH 12 solutions. Their pK_a values are, admittedly, much lower than 12, but this pH is necessary for P and 2,4-DMP. For example, after 1 h at room temperature in a pH 12 solution, the derivatization yield for PCP had decreased by 10–30%, whereas a *ca.* 90% loss was found after 4 h.

Volume ratio in the two-phase reaction. Normally, 500 μl of an aqueous sample, 100 μl of a 30 mg/ml solution of TBABr and 600 μl of a 0.1 mg/ml solution of dansyl chloride in dichloromethane were vortex mixed for 2 min, and 500 μl of the organic layer were brought onto an amino SPE column for removal of the excess of reagent. In this way a sensitivity of *ca.* 0.1–0.4 ng/ml can be achieved. To obtain detection limits lower than 0.1 ng/ml, another procedure was necessary, *e.g.*, preconcentration on a short C_{18} precolumn. For apolar chlorophenols this is a reliable approach; polar phenols (2-NP, 4-NP and P), however, break through almost immediately. An alternative is to increase the aqueous phase volume in the two-phase reaction from 500 to 2000 μl, the TBABr solution volume from 100 to 300 μl and the dansyl chloride solution volume from 500 to 800 μl. For the relatively polar phenols (2- and 4-NP, P and 2-CP) this approach worked well and a gain in sensitivity by a factor of about four was obtained. However, for the more apolar compounds such as DMP, CMP and DCP (see Fig. 1), large interfering peaks appeared in the chromatogram. Therefore, all apolar phenols (DMP, DCP, CMP, TCP and PCP) were preconcentrated from 3.0 ml of an acidified aqueous sample on a C_{18} precolumn, as described [20]. The C_{18} precolumn was desorbed with 3 ml of dichloromethane and the eluate was evaporated under a stream of nitrogen. To the residue 600 μl of the dansyl chloride solution in dichloromethane, 500 μl of water (pH 12) and 100 ml of the TBABr solution were added and the normal derivatization procedure was followed. It is an advantage that the higher chlorinated phenols are in contact with the pH 12 solution for only a relatively short time.

Photochemical conversion of dansylated phenols

As has been described above, photochemical conversion of dansyl derivatives into more strongly fluorescent products can be used conveniently in a postcolumn LC system [11]. Photochemical conversion is important, because dansylated chlorophenols containing two or more chlorine atoms exhibit little native fluorescence, especially when the chlorine atoms are in the *ortho* position to the phenolic OH group. After LC separation the dansylated phenols are irradiated by a mercury lamp, and two main products are formed: the non-fluorescent chlorophenol and the highly fluorescent dansyl hydroxide and/or dansyl methoxide group.

When analysing a mixture of dansylated chloro- and nitrophenols (present at the same molar concentration level) by means of LC and postcolumn photolysis, surprisingly we observed that the peak heights of the mononitrophenols were about four times lower than those of the chlorophenols, and the dinitrophenols (DNP, DNMP, dinoseb and dinoterb) gave no peak at all. This aspect was investigated further by injecting relatively high concentrations (*ca.* 1 μg/ml) onto an LC column, with subsequent photolysis and UV monitoring, isolating the irradiated peaks and reinjecting a fraction of these peaks in the same system. In principle, each peak should

contain the following analytes: dansyl hydroxide and/or methoxide and, if still present, the non-decomposed dansylated phenol. The photochemical conversion can be calculated by comparing the peak height of the dansylated phenol peak in the first chromatogram with the peak in the same position after the second analysis. For several chlorophenols a 90–95% yield was observed, as against 30–40% for the mononitrophenols. For all dialkylphenols tested (2,3-, 2,4-, 2,5-, 3,4- and 3,5-dimethylphenol and 2-isopropyl-5-methylphenol), a photochemical conversion of 100% was found. This means that dansylated chloro- and dialkylphenols are very efficiently decomposed after irradiation for only 5 s, whereas in the same time the dansylated mononitrophenols are converted into fluorescent products to the extent of only 30–40%. Changing the irradiation time in the PTFE coil from 5 to 15 s did not improve the results. In fact, the peak heights of the dansylated mononitrophenols remained about the same, while all dansylated chlorophenol peak heights were 30–50% lower, probably owing to photochemical breakdown. The use of a more transparent quartz coil instead of a PTFE coil also did not improve the photochemical conversion of the nitrophenols, the peak-height ratio of chlorophenols to nitrophenols remaining about the same. As a quartz coil is more expensive and fragile than a PTFE capillary, all further studies were carried out with the standard PTFE capillary.

At a pH of 8, ion-pair extraction of dinitrophenols into chloroform or dichloromethane could easily be carried out, but no reaction with either dansyl chloride or laryl chloride was observed. It seems likely that the negative charge of the phenolate anion is strongly delocalized by the nitro groups. The nucleophilic character of the phenolate anion is reduced and, consequently, the reactivity. In other words, the present method is not suitable for dinitro-substituted phenolic compounds.

Chemiluminescence detection

Peroxyoxalate CL detection is usually combined with acetonitrile–water mobile phases. In that case, the oxalate, 2-NPO and hydrogen peroxide dissolved in acetonitrile can be added to the mobile phase without mixing problems. In this study, methanol was necessary as mobile phase modifier, because in postcolumn photochemistry acetonitrile is not compatible with a PTFE coil. Radicals are formed and rapid leakage of the PTFE coil will occur. Using the quartz coil described above, acetonitrile could be compared with methanol as mobile phase modifier. The signal-to-noise ratio for a dansylated phenol in acetonitrile–water mixtures was three times better than in methanol–water, but not all the peaks could be separated with an acetonitrile–water gradient; therefore, methanol–water was used.

When combining gradient elution with peroxyoxalate CL detection, care must be taken that both solvents contain an equal amount of buffer [21]. The mobile phase will then continuously contain the same concentration of imidazole buffer, an important parameter in CL detection. Furthermore solvents should be filtered and degassed very carefully, because air bubbles and microprecipitates in the mixing chamber will cause baseline disturbances when performing a gradient at trace-level sensitivity.

The imidazole buffer was varied both in concentration (1 and 10 m*M*) and pH (6 and 7.5), two of the most critical parameters in peroxyoxalate CL detection in LC systems [22,23]. With dansylated phenol as the test compound, the imidazole concentration gave a maximum in the CL signal-to-noise ratio at about 2.5 m*M*, and the pH at 7.0.

The oxalate (2-NPO, 5 m*M*) and hydrogen peroxide (50 m*M* in acetonitrile) were used as described in an earlier paper [24]. The addition of the 2-NPO and hydrogen peroxide mixture in acetonitrile (at 0.3 ml/min) to the methanol–water column eluate (0.5 ml/min) never led to any mixing or precipitation problems.

The ATTO AC 2220 CL detector is one of the few commercially available CL detectors for LC. Its photomultiplier tube is operated at a standard voltage of 1000 V. Unfortunately, in our system the noise was too large for us to be able to use the whole range of attenuation settings on the ATTO AC 2220. Therefore, our electronic workshop changed the photomultiplier power supply in such a way that it could be manually adjusted between 600 and 1000 V. Throughout this study a voltage of 700 V was applied. At this photomultiplier voltage the complete range of attenuation settings could be used.

Comparison of fluorescence with CL detection

The two detection modes were compared by injecting a mixture of four dansylated phenols. Fluorescence detection was carried out with an Perkin-Elmer LS-2 fluorescence detector equipped with a xenon lamp (excitation wavelength 340 nm, 470 nm, time constant 1 s) and CL detection was performed as described under Experi-

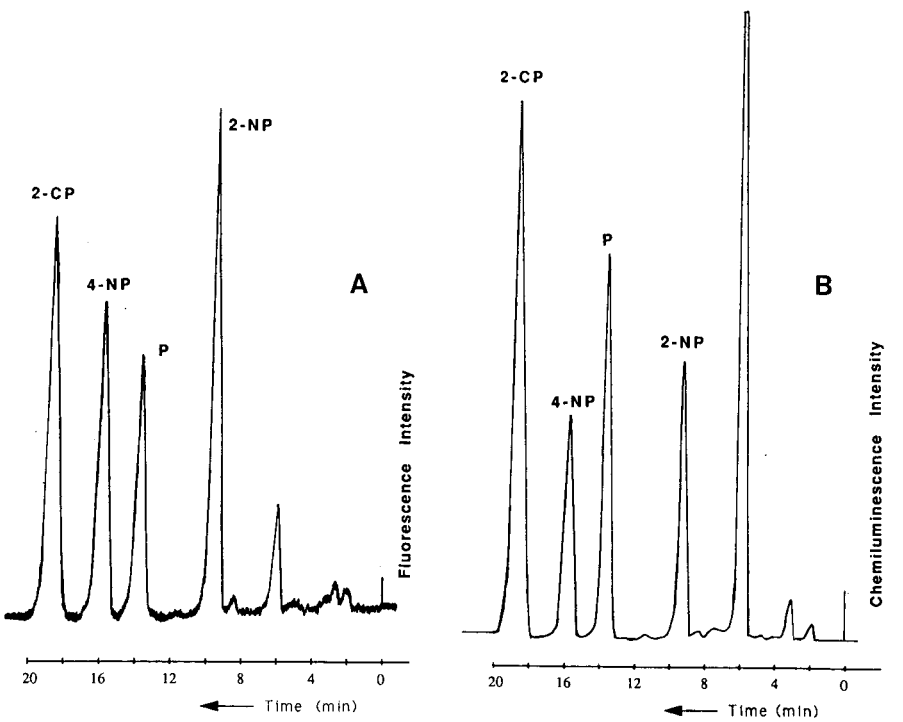

Fig. 2. (a) LC with fluorescence detection of a mixture of four dansylated phenols (see Fig. 1); 4 ng (phenol and 2-chlorophenol) and 20 ng (mononitrophenols) injected. Detector, Perkin-Elmer LS-2 (λ_{exc} = 340 nm, λ_{em} = 470 nm; response time, 2 s); mobile phase, A–B (75:25) flow-rate, 0.5 ml/min. For derivatization and further details, see Experimental. (B) LC with CL detection of the same dansylated phenols as in (A). Detector, ATTO AC-2220; attenuation , 200; noise is obtained at attenuation 2 (100 times more sensitive); mobile phase A–B (75:25); flow-rate, 0.5 ml/min. For derivatization and further details, see Experimental.

mental. As can be seen from Fig. 2, the signal-to-noise ratio with CL detection is about two orders of magnitude better than that with fluorescence detection. Another interesting point is that the peak-height ratio of nitrophenols to chlorophenols is slightly higher with fluorescence detection. This may be due to the excitation lamp irradiating the dansyl derivatives during analysis.

Linearity and detection limits

The linearity of the complete system (derivatization, photolysis and CL detection) was measured for a mixture of four dansylated phenols, 2- and 4-NP, P and 2-CP, in the range 5–500 ng/ml. In all instances the linearity was good ($n = 9$, $r = 0.996$–0.998).

The limits of detection for normal derivatizations (500 μl of aqueous phenolic solution) were 0.4 and 0.05 ng/ml for phenol and 2-chlorophenol, respectively and 0.4 ng/ml for 2-NP and 4-NP. For the higher chlorinated phenols slightly higher limits of detection were obtained than for 2-CP, ranging from 0.1 ng/ml for 2,4-DCP to 0.2 ng/ml for PCP. When using a larger aqueous phase volume or preconcentration via a precolumn, the limits of detection were improved accordingly (see Table I). In some instances they were influenced by the appearance of small peaks due to the formation of derivatization side-products (especially with P and DMP), but in almost all instances the required detection limit of 0.1 ng/ml was achieved.

Tap and river water samples

Tap and river water were spiked with phenols at the 0.5–2.5 ng/ml level. For the four early-eluting phenols (2- and 4-NP, P and 2-CP), the water was adjusted to pH 12 with 1 *M* sodium hydroxide solution and 2000 μl were derivatized as described under Experimental. For the five late-eluting phenols (DMP, CMP, DCP, TCP and PCP) the water was acidified to pH 3 and 3.0 ml were preconcentrated on a C_{18} precolumn. A larger preconcentration volume, *e.g.*, 10–20 ml, is still below the breakthrough volumes of these relatively apolar phenols and will probably result in lower

TABLE I

LIMITS OF DETECTION IN ng/ml (SIGNAL-TO-NOISE RATIO = 3) FOR SEVERAL PHENOLIC COMPOUNDS

Dansylated derivative of	Aqueous sample volume[a]		
	500 μl	2.0 ml	3.0 ml
2-Nitrophenol	0.4	0.1	
4-Nitrophenol	0.4	0.1	
Phenol	0.4	0.1	
2-Chlorophenol	0.05	0.01	
2,4-Dimethylphenol	0.5		0.1
3-Methyl-4-chlorophenol	0.1		0.02
2,4-Dichlorophenol	0.1		0.02
2,4,6-Trichlorophenol	0.1		0.03
Pentachlorophenol	0.2		0.04

[a] For procedures, see Experimental.

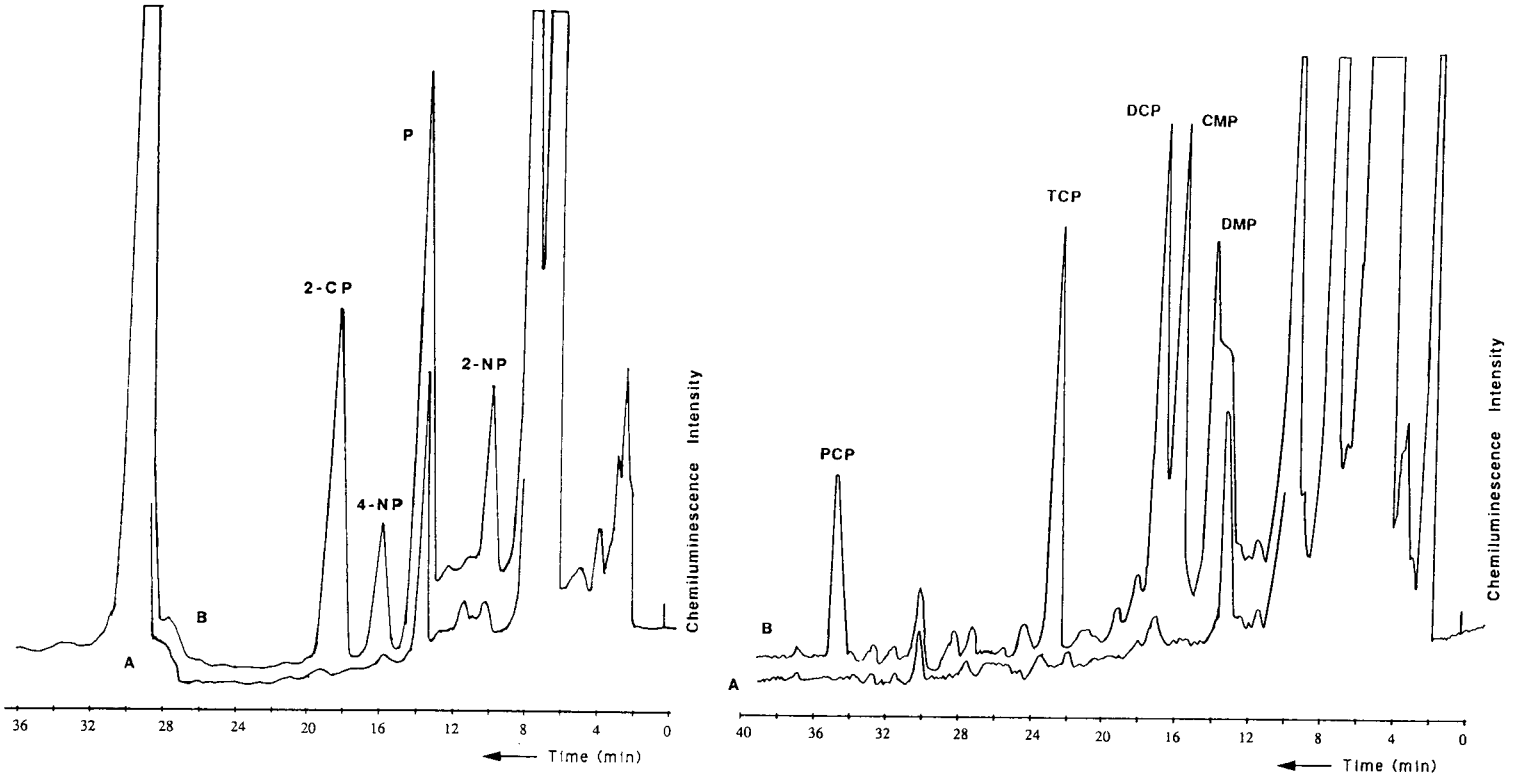

Fig. 3. LC with CL detection of Rhine water, (A) unspiked and (B) spiked with 0.5 ppb each of phenol and 2-chlorophenol and 2.0 ppb of mononitrophenols. The peak coeluting with dansylated phenol in the chromatogram of unspiked Rhine water is caused by derivatization side-products. Attenuation, 2; time constant, 5 s; mobile phase, A – B (75:25); flow-rate, 0.5 ml/min. For derivatization and further details, see Eperimental.

Fig. 4. Gradient LC with CL detection of Rhine water, (A) unspiked and (B) spiked with 0.8 ppb of phenols. Attenuation, 5; time constant, 2 s. Gradient: 0–6 min, 85% A (15% B); 6–20 min, 85–95% A (15–5% B); 20–35 min, 95% A (5% B); 35-36 min, 95–85% A (5–15% B); flow-rate, 0.5 ml/min. For derivatization and further details, see Experimental.

concentration detection limits. After desorption with dichloromethane and gentle evaporation of the solvent with a stream of nitrogen, the derivatization was carried out as described under Experimental. Fig. 3 shows a chromatogram of a Rhine water sample (spiked with 2- and 4-NP, P and 2-CP) and demonstrates the high sensititivy and selectivity of the detection system. Fig. 4 shows a gradient elution chromatogram of river Rhine water spiked with five apolar phenolic compounds (DMP, CMP, DCP, TCP and PCP).

CONCLUSION

A rapid and relatively simple method for the sensitive and selective determination of phenolic compounds is described. Only 2–3-ml aqueous samples are used and laborious liquid–liquid extraction becomes superfluous. The two-phase dansylation procedure described earlier for tri-, tetra- and pentachlorophenols by means of LC with fluorescence detection can be extended to relatively polar phenols such as phenol, mononitrophenols and 2-chlorophenol. However, derivatization is not successful for dinitrophenols in the two-phase ion-pair extraction dansylation. This may be ascribed to the strongly electron-withdrawing nitro groups which cause a strong decrease in electron density on the phenolic oxygen, leading to a very low reactivity. A major improvement of the derivatization scheme is the removal of the excess of dansyl chloride by an amino SPE column, which results in much cleaner chromatograms. In principle, this approach is amenable to the removal of other reagents suitable for primary amines, such as 9-fluorenyl methylchloroformate (FMOC), fluorescein isothiocyanate (FITC), 2,4-dinitrofluorobenzene (DNFB), 4-dimethylamino-4′-azobenzenesulphonyl chloride (dabsyl chloride) and lissamine rhodamine B sulphonyl chloride (laryl chloride). It may even be extended to reagents for other functional groups such as thiols, aldehydes, alcohols and carboxylic acids if suitable cartridges are available.

The postcolumn photochemical reaction for dansylated phenols leads to the formation of the fluorescent dansyl hydroxide and/or methoxide [5]. It should be emphasized that the system described above shows a nearly universal response for all chloro- and alkylphenols if molar concentrations are used. However, the photochemical conversion of dansylated mononitriphenols is about four times lower than that for dansylated chlorophenols and the detection limits are accordingly higher. The postcolumn photochemical reaction can easily be combined with peroxyoxalate CL detection, yielding detection limits which are about 100 times lower than with fluorescence detection. Linearity is good for all phenols from 5 to 500 ng/ml and the detection limits are at sub-ppb levels.

The applicability of the present system for the trace analysis of other phenolic compounds, *e.g.*, drugs and their metabolites, will be investigated in the near future.

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Practical strategy for the analytical separation of enantiomers by high-performance liquid chromatography

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ABSTRACT

When a pharmacologically active compound containing a chiral centre is identified as a potential development cadidate, it is considered essential to define the pharmacological properties of the pure enantiomers. High-performance liquid chromatography has a critical role in a pharmaceutical research department by providing sensitive measurements of optical purity.

To react effectively to the demands of a large chemistry team, a strategy has been developed. Key racemates are selected for method development during the early stages of each project team's effort. Separation methods for these compounds are identified from a consideration of such factors as physicochemical data, pharmacological potency etc. The methods are evaluated and optimised separations are established to measure optical purity.

Separation methods for analogous molecules synthesised later in a project are predictable from this strategic assessment, and the need for significant extra method development is avoided.

INTRODUCTION

Chiral separations by high-performance liquid chromatography (HPLC) are difficult because unlike achiral compounds, enantiomers have the same physical properties except for their rotation of plane polarised light. It is often held that the direct separation of optical isomer requires a minimum of three simultaneous interactions with a chiral selector, at least one of which is stereochemically controlled [1]. Other workers have reported that a two-point interaction between two chiral structures is sufficient for mutual chiral recognition, provided each of these structures simultaneously contacts a third non-chiral species, *e.g.* a solid surface [2].

A strategy is described which rationalises the complexities of analytical chiral method development using several representative molecules from a series of racemic thromboxane antagonists as examples.

Seperation methods were developed to measure the optical purity of the enantiomers of each compound to levels defined by pharmacological data.

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EXPERIMENTAL

Materials

Compounds 1–3 (see Table I) were synthesised at Alderley Park. Hexane, propan-2-ol, methanol and acetonitrile were HPLC grade (Fisons, Loughborough, UK). Ethanol was re-rectified absolute and water was doubly distilled from glass. Sodium dihydrogenphosphate and disodium hydrogenphosphate were AnalaR Grade (BDH, Poole, UK). Bovine serum albumin was Fraction V (Sigma) and β-cyclodextrin was purchased from Aldrich (U.K.).

Spherisorb S50DS, S5CN, LiChrosorb 10-μm Diol and covalent Pirkle (*R*)-3,5-dinitrobenzoylphenylglycine columns (all 25 cm × 4.6 mm I.D.) were purchased from Hichrom (Reading, U.K.). A 25 cm × 4.6 mm I.D. Daicel OD column and a 10 cm × 4 mm I.D. Chiral AGP column were supplied by J. T. Baker (Hayes, U.K.) and a 25 cm × 4.6 mm I.D. Cyclobond I column was purchased from Technicol (Stockport, U.K.).

STRATEGY

The strategy is based on a sequential assessment of solute structure and physical data, analytical considerations, chiral selectors, eluent and selector combinations and finally method suitability.

Structure and physical data

A full knowledge of solute structure and reactivity, octanol–water partition coefficient (log *P*) data, pK_a, solubility, UV and stability is required.

Analytical considerations

The physical data are used to develop an achiral HPLC system which is used to measure the solute purity, and to check the stability of the solutes in the selected chiral systems. Potential chiral eluent additives must be optically pure for sensitive measurements to be achieved and the UV spectra of the solute and selected additives are compared to determine the absorbance of the compounds against the background. Other detectors may be considered *e.g.* fluorescence.

Chiral selectors

The interactive sites on the solute and the size(s) of the molecule and its functional groups are assessed. Chiral stationary phases or chiral eluent additives which favour this assessment are chosen, *e.g.* a hydrophobic, ionic selector interacts with an ionic hydrophobic solute. Indirect methods which modify the interactive properties of a solute by achiral derivatisation are also identified, which will allow previously unfavoured selectors to be used. Detectability can be enhanced simultaneously if a strong UV chromophore is incorporated.

Eluent–selector combinations

Chiral stationary phases (CSPs). The eluent polarity is chosen to favour the predicted solute–selector interactions without compromising solute solubility, *e.g.* electrostatic and hydrophobic interactions between a selector and an ionic solute are

most effective when the compound is dissolved and eluted in a polar ionising eluent–water. Alternatively separations relying on weak interactions, *e.g.* H-bonds will be favoured in dry, low-polarity eluents.

Chiral eluent additives. The eluent is chosen to enhance the solute–selector interactions, but the solubility of both the solute and the additive must be maintained. Solutes of varying log *P* will dissolve in a range of eluent polarities by choosing the selector carefully, *e.g.* hydrophobic solutes will dissolve in aqueous eluents via complexation with polar selectors *e.g.* cyclodextrins. Applications where polar aminoalcohols are dissolved in apolar eluents after ion pairing with hydrophobic chiral counter ions have been reported [3].

Method suitability

A chromatographic optical purity method is used to measure the amount of a minor enantiomer in resolved material to a level which is usually defined by the pharmacological efficacies of each enantiomer. When methods are identified, they are evaluated against these criteria. If new information implies new target measurements, alternative methods identified during the strategic assessment can be evaluated quickly.

EXAMPLE

Structure and physical data

Three compound were selected for method development because they reflected the range of the log *P* values in a series of racemic thromboxane antagonists (Table I). UV spectra were recorded for each compound.

Analytical considerations

The analytical purity of each solute was measured as >99% using eluents of methanol–water modified with trifluoroacetic acid on a Spherisorb S50DS column. The solutes were allowed to stand in the selected eluents (see below) for 1–2 days and rechromatographed against freshly prepared standards without evidence of decomposition. The optical purity of the two selected chiral additives β-cyclodextrin and bovine serum albumin (BSA) was >99.9%, but the UV absorbance of the protein precluded its use as a selector for compound 3 (Fig. 1).

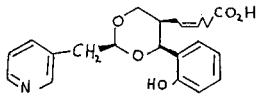
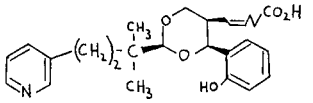
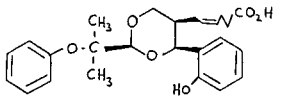
Chiral selectors

α_1 - Acid glycoprotein (AGP) and bovine serum albumin (BSA) were identified as selectors for charged molecules containing hydrophobic moieties. AGP has been used for a range of drugs [4] and several high affinity sites on BSA for carboxylic acids have been reported [5]. Certain solute groups (*e.g.* aryl) are correctly sized to fit into the cavity of a third selector, β-cyclodextrin. Enantioselectivity depends on the degree of inclusion and H-bond interactions between secondary hydroxyls at the cavity mouth and excluded solute groups [6].

Non-aqueous chiral ion-pair systems were unfavourable. Ion-pair formation between the solutes and a chiral counter ion (quinine) would occur, but the lack of an H-bond acceptor β to the acid would preclude a secondary interaction with the C9-OH in quinine.

TABLE I

STRUCTURES AND PHYSICAL DATA

Compound		log P	pK_a acid	S^a (mg/ml)	S^b
1		3.5	4.9	1.0-2.0	1.0
2		5.4	4.9	1.0-2.0	0.5-1.0
3		6.0	4.9	2.0-5.0	0.1-0.5

[a] Solubility in propan-2-ol.
[b] Solubility in sodium phosphate solution pH 7.0.

It was recognised that some unfavoured selectors could be utilised by enhancing the H-bond and charge transfer characteristics of the solutes and a chemical derivatisation to form the α-naphthylamides [7] was defined. The method was not evaluated initially but selective interactions with (*R*)-3,5-dinitrobenzoylphenylglycine [8] (Pirkle) and cellulose tris (3,5-dimethylphenylcarbamate) [9] (Daicel OD) were predicted.

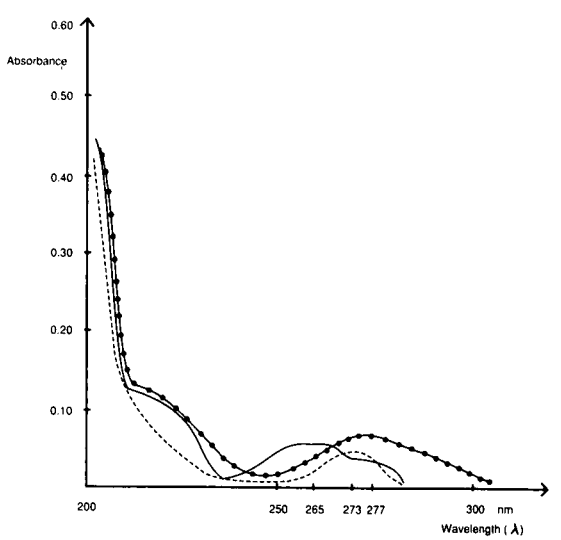


Fig. 1. Comparison of UV spectra of BSA (●—●), compound 2 (———) and compound 3 (– – –).

Eluent–selector combinations

Chiral AGP, Cyclobond I, Pirkle and Daicel OD columns were identified as chiral stationary phases. Bovine serum albumin and β-cyclodextrin were identified as chiral eluent additives.

Chiral stationary phases. A buffered aqueous eluent (pH 7.0) was used with Chiral AGP to ionise the compounds and the protein (pI = 2.7) and to promote an electrostatic effect. Because the carboxylates were water soluble, the amounts of organic modifier (propan-2-ol) could be reduced and the hydrophobic solute–protein interactions were favoured.

Using Cyclobond I, the compounds were dissolved as anions in a buffered aqueous eluent (pH 7.0). The need for an organic solvent (acetonitrile) to force elution was reduced and the hydrophobic cavity–solute interactions were enhanced.

Dry, low-polarity mixtures of alcohols with hexane were identified for separation of the α-naphthylamides using Pirkle and Daicel OD columns. Weak interactions (H-bond, charge transfer) are favoured in apolar media, while solute solubility is maintained through a higher log P.

Chiral eluent additives. Ionising, solvating aqueous eluents at pH 7.0 were selected for use with bovine serum albumin and β-cyclodextrin. The flexibility of the additive technique was exploited by using a range of column polarities (LiChrosorb Diol → Spherisorb S50DS) to identify eluents containing very high amounts of water, in order to dissolve the chiral additives at the high concentrations which favour complexation. When necessary, propan-2-ol and acetonitrile were used as organic modifiers for the protein and cyclodextrin systems, respectively.

TABLE II

CHROMATOGRAPHIC SEPARATION DATA

α = Separation factor; R_s = resolution. A = 50 mM NaH_2PO_4–50 mM Na_2HPO_4 (1/1) pH 7.0. B = 10 mM NaH_2PO_4–50 mM Na_2HPO_4 (1/1) pH 7.0.

Method	Compound 1		Compound 2		Compound 3	
	α	R_s	α	R_s	α	R_s
Chiral AGP[a]	1.10	0.7	1.10	0.7	1.40	0.8
Cyclobond I[b]	1.30	0.9	1.08	0.6	1.06	0.2
Bovine serum albumin[c]	1.10	0.9	1.20	0.9	Not evaluated	
β-Cyclodextrin[d]	1.40	1.7	1.00	0.0	1.00	0.0
Pirkle[e,g]	Not evaluated		Not evaluated		1.10	0.8
Daicel OD[f,g]	Not evaluated		Not evaluated		1.40	1.8

[a] B–propan-2-ol (85:15).
[b] A–acetonitrile (90:10).
[c] A–propan-2-ol (90:10) + 3 mg/ml bovine serum albumin/Spherisorb S5CN.
[d] A–acetonitrile (90:10) + 15 mg/ml β-cyclodextrin/Spherisorb S5CN.
[e] Hexane–propan-2-ol (80:20).
[f] Hexane–ethanol (85:15).
[g] Compound 3 chromatographed as the α-naphthylamide derivative.

Method suitability

Optical purity measurements to levels of 1–2% were targeted to support some early pharmacological work on the solute enantiomers. The direct methods established for the compounds met these criteria (Table II).

The selection of compound 3 for development coupled with new pharmacological data indicating a 1:1000 efficacy ratio for the enantiomers demanded more stringent chiral methodology. The resolution of compound 3 was insufficient for measurements below 1–2% (Fig. 2) and a separation of the α-naphthylamide derivative on Pirkle and Daicel columns was evaluated (Table II). The optimum separation was identified (Fig. 3) and used to measure <0.2% of the (−)-enantiomer in the (+)-enantiomer (Fig. 4).

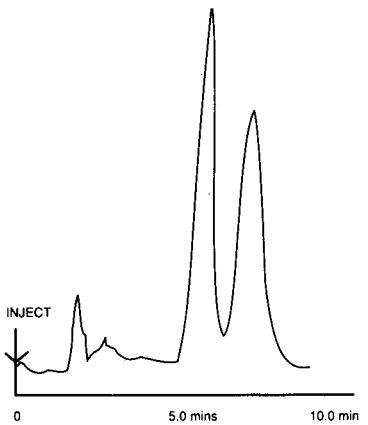


Fig. 2. Preliminary method for compound 3. Column: 10.0 cm × 4.0 mm I.D. Chiral AGP. Eluent: [10m*M* Na_2HPO_4–10 m*M* NaH_2PO_4 (1/1) pH 7.0]–propan-2-ol (85:15). Flow-rate: 0.9 ml/min. Detection: 273 nm.

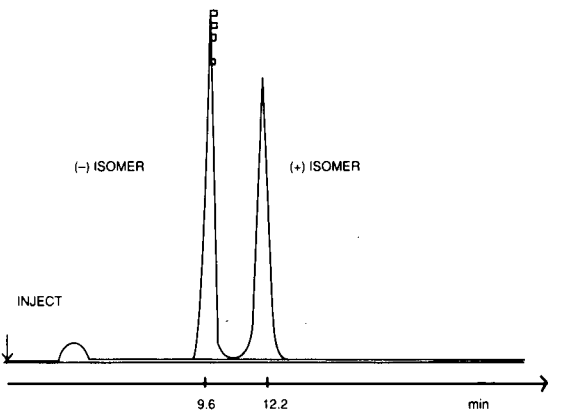


Fig. 3. Final method for compound 3 (α-naphtylamide) Column: 25.0 cm × 4.6 mm I.D. Daicel OD. Eluent: Hexane–ethanol (85:15). Flow-rate: 1 ml/min. Detection: 222 nm.

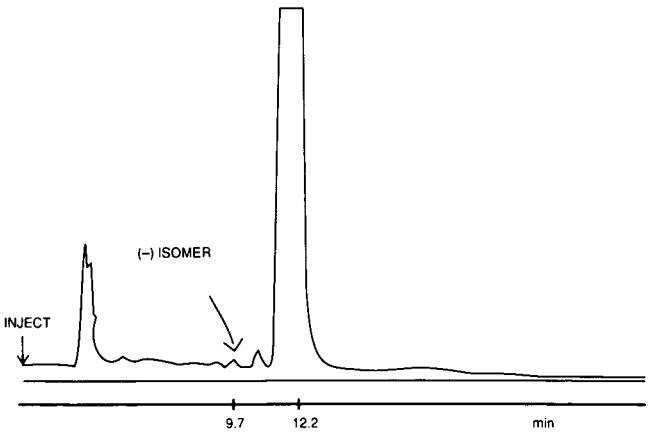


Fig. 4. Optical purity measurement. Column: 25.0 cm × 4.6 mm I.D. Daicel OD. Eluent: Hexane–ethanol (85:15). Flow-rate: 1 ml/min. Detection: 222 nm.

CONCLUSIONS

A strategy to develop chiral HPLC methods has been described. Separations to measure the optical purity of single enantiomers were established which met changing measurement criteria which were defined by pharmacological data. The selection of an enantiomer as a development candidate was not delayed by additional method development.

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CHROMSYMP. 2257

Influence of chemical structure of tricyclic tertiary dimethylamines on chiral separation by reversed-phase high-performance liquid chromatography after derivatization with (−)-menthylchloroformate

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ABSTRACT

In the present study (−)-menthylchloroformate was used as a chiral derivatizing agent for promethazine, trimeprazine, trimipramine and N-(2-dimethylaminopropyl)iminodibenzyl. (−)-Menthylchloroformate reacted with the tertiary dimethylamine moiety in these tricyclic antihistamines and antidepressants, resulting in the formation of diastereoisomers. Owing to the reaction conditions, during the derivatization with (−)-menthylchloroformate, the possibility of racemization had to be established. For this purpose different ratios of (+) and (−)-promethazine were prepared. Enantiomeric separation of these mixtures took place on a Chiral α AGP column or, after derivatization with (−)-menthylchloroformate, on a C_{18} column. The results from these two independent separation systems were compared with trace racemization. No racemization was found during the experiments. To study the effects of changes in the molecular structures of the tertiary dimethylamines on the chromatographic behavior of the derivatization products, four tertiary dimethylamines [promethazine, trimipramine, trimeprazine and N-(2-dimethylaminopropyl) iminodibenzyl] were derivatized and analyzed. With these amines the effects on resolution and capacity factor of replacing a phenothiazine ring by an iminodibenzyl ring or insertion of a carbon molecule between the chiral centre in the chain and the place where (−)-menthylchloroformate reacts were studied. Not only the distance between the chiral centres in the diastereoisomers (a longer distance caused less resolution and higher capacity factors) but also the kind of ring influenced resolution and capacity factor. Finally, the influence of eluent composition on resolution and capacity factor was studied. Three different mixtures of methanol and acetic acid were tested. More acetic acid in the eluent caused a better resolution and higher capacity factor. The higher capacity factor, however, resulted in unacceptable retention times.

INTRODUCTION

The recent interest in enantioselectivity in the pharmacological behavior of pharmaceutics is causing a growing need for enantioselective bioanalytical methods. High-performance liquid chromatography (HPLC) is a method with numerous applications for the quantitation of drugs in biological fluids. With direct chiral HPLC methods enantiomeric separation is possible, at least for pure drugs. However, most

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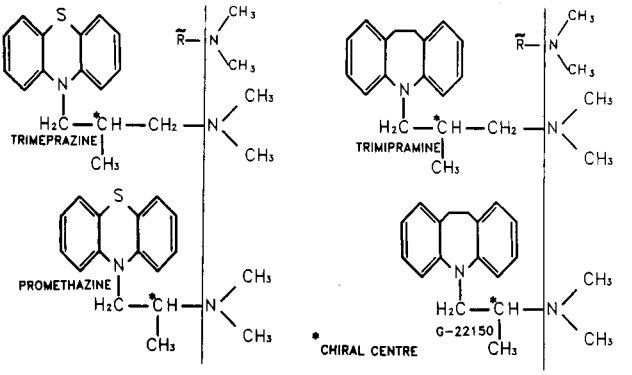

Fig. 1. Structures of the four tertiary dimethylamines.

chiral stationary phases are easily impaired by endogenous compounds from biological matrices such as plasma or urine.

A different approach, the indirect one, is the use of an optically pure derivatizing agent which results in the formation of diastereoisomers. Diastereoisomers have different chemical and physical properties which allow, at least in theory, separation on non-chiral HPLC systems. For the derivatization reaction a suitable functional group in the racemic drug is required. For various easily accessible groups, such as alcohols and primary and secondary amines, suitable derivatization agents exist [1]. Another functional group, present in many pharmaceuticals, is the tertiary amine. For this group only a few derivatization reactions have been described [2].

We recently used (−)-menthylchloroformate to derivatize tertiary dimethylamines, using promethazine as an example [3]. In this paper we have further evaluated the potential of this derivatizing agent. Racemization, due to the applied reaction conditions, was checked for promethazine by comparing the results of the derivatization with the results of an independent direct separation system.

The influence of changes in the chemical structure of the tertiary amine on resolution and capacity factor was also studied. Four amines with comparable structures consisting of a phenothiazine or an iminodibenzyl ring, and a 2-(dimethylamino)-2-methylethyl or a 3-(dimethylamino)-2-methylpropyl side-chain were analyzed. Their structures are given in Fig. 1 and the derivatization reaction with (−)-menthylchloroformate is depicted in Fig. 2.

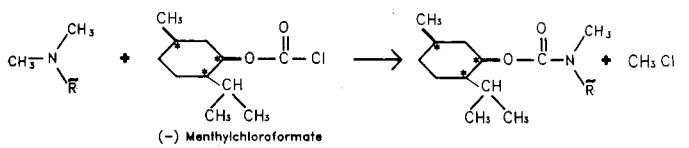

Fig. 2. Derivatization reaction of the tertiary dimethylamines with (−)-menthylchloroformate.

EXPERIMENTAL

Chemicals

Racemic tertiary dimethylamines. Promethazine hydrochloride was obtained from Brocacef (Maarsen, The Netherlands) and was of Ph. Eur. quality. Trimipramine maleate was from Centrachemie (Etten-Leur, The Netherlands). Trimeprazine tartrate came from May & Baker (Manchester, U.K.), and was of B.P. quality.

N-(2-Dimethylaminopropyl)iminodibenzyl, "G-22150", was synthesized according to a modification of the method in ref. 4 with iminodibenzyl from Janssen (Beerse, Belgium) and 1-dimethylamino-2-propylchloride from Aldrich-Chemie (Steinheim, Germany). The reaction product was tested and its identity verified with mass spectrometry, electron impact (EI) and chemical ionization (CI), and ^{1}H nuclear magnetic resonance. The structures of the tertiary amines are given in Fig. 1.

Other reagents. Acetonitrile and methanol were from Westburg (Leusden, The Netherlands) and were of HPLC grade. Triethylamine was purchased from Janssen and was of analytical grade. The (−)-methylchloroformate {$[\alpha]_D^{18}$: 80° (1 g per 100 ml chloroform)} was obtained from Aldrich-Chemie. Acetic acid, diethyl ether, toluene, xylene and sodium dihydrogenphosphate 1-hydrate, all of analytical grade, were from E. Merck (Darmstadt, Germany).

Chromatographic equipment

A reversed-phase HPLC (RP-HPLC) system with a 150 × 4.6 mm I.D. stainless-steel column, filled with Nucleosil C_{18} 5 μm (Macherey Nagel, Düren, Germany) was used to separate the diastereoisomers formed during derivatization. For the direct chiral separations a Chiral α AGP column, 100 × 4 mm I.D. (ChromTech, Stockholm, Sweden) was used.

In the experiments to check racemization during or after the derivatization, the solvent delivery system was a 2150 LKB HPLC pump (Pharmacia LKB Biotechnology, Uppsala, Sweden), injections were made by a 710 A WISP (Waters, Milford, MA, U.S.A.) and the UV detector was an HP 1040A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA, U.S.A.) operating at 258 nm. For recording and integrating the chromatograms a C-R3A Chromatopac (Shimadzu, Kyoto, Japan) was used.

In the experiments to study the influence of the chemical structure of the tertiary dimethylamine on resolution and capacity factor, the same RP-HPLC stationary phase was used.

The solvent delivery system here was an M 6000 A (Waters). The injector was a Model 7125 manual loop injector equipped with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.) and the detector was a Spectraflow 757 UV detector (Kratos, Ramsey, NJ, U.S.A.) at 252 nm. For recording the results a BD 40 recorder (Kipp, Delft, The Netherlands) was used.

Derivatization

The derivatization of the tertiary dimethylamines was carried out according to a modification of the method in ref. 3. To a 250-μl sample of a 0.1 mg/ml solution of the tertiary dimethylamine in acetonitrile were added 500 μl of 10% (v/v) (−)-menthylchloroformate in acetonitrile and 25 μl of triethylamine. The solution obtained

was heated for 1.5 h at 70°C. After cooling the mixture to room temperature, a 20-μl sample was injected into the RP-HPLC system.

Check for racemization

Solutions of (+)- and (−)-promethazine were prepared in acetonitrile (50 μg/ml) and mixed to give samples with various ratios of the (+) and (−) enantiomers. The enantiomers of promethazine were obtained by crystallization [5] and the enantiomeric purity of the enantiomers thus obtained was verified with the use of a Chiral α AGP stationary phase.

The samples were analyzed by both a direct and an indirect HPLC method under the following conditions.

Direct method: no derivatization, enantiomeric separation on a chiral α AGP with an eluent consisting of 11% acetonitrile and 89% 0.1 *M* sodium dihydrogen phosphase in distilled water, pH 6.0. Eluent flow-rate 0.9 ml/min.

Indirect method: derivatization with (−)-menthylchloroformate and the separation of the formed diastereoisomers on a C_{18} HPLC column with an eluent consisting of 75% acetonitrile and 25% 0.1 *M* acetic acid in distilled water, pH 2.9. Eluent flow-rate 1.0 ml/min.

The percentages of the (+) area and the (−) area relative to the total area [(+) + (−)] were calculated.

Influences of chemical structure of the tertiary dimethylamines on capacity factors and resolutions of the derivatization products in three eluent compositions in RP-HPLC

The capacity factors of the first eluting diastereoisomers were used. The resolution between the two diastereoisomers was measured according to ref. 6. The effects of changes in ring and chain structure on resolution and capacity factor were calculated. Eluent compositions used were mixtures of methanol and 0.1 *M* acetic acid (v/v) with ratios (a) 90:10; (b) 85:15; and (c) 80:20. The flow-rate for all three eluent compositions was 1.0 ml/min.

RESULTS AND DISCUSSION

Check for racemization

To check for racemization during or after the derivatization reaction two methods were compared. The indirect method, derivatization with (−)-menthylchloroformate, was compared with a direct method, separation on a Chiral α AGP column.

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF THE INDIRECT METHOD AND THE DIRECT METHOD FOR RACEMIC PROMETHAZINE

Method	Enantiomer	Retention time (min)	Resolution	Plate number
Indirect	−	17.97	2.31	6580
	+	20.28		5167
Direct	+	18.01	1.67	1072
	−	21.68		1554

In Table I the chromatographic characteristics of the indirect and the direct method are compared for racemic promethazine.

The inversion of the elution order for the promethazine enantiomers between the two methods is advantageous for the assessment of optical purity. In practice, this means that a small amount of (+) in a large amount of (−) can be determined more accurately with the direct method, whereas the indirect method is more accurate when a small amount of (−) in a large amount of (+) is to be determined. The results of the experiments with the mixtures containing different ratios of (+)- and (−)-promethazine are summarized in Table II.

The lines for the percentage (+) in the sample could be calculated for the two methods according to the equation $y = ax + b$ where y is the percentage of (+) analyzed and x is the percentage of (+) attempted. Direct method: $y = 0.980\ x - 0.104$ ($r = 0.9990$); indirect method: $y = 0.992\ x + 1.848$ ($r = 0.9993$); indirect/direct: $y = 0.998\ x + 2.877$ ($r = 0.9986$).

The linearity of the lines with a slope near 1 showed that no racemization occurred during these experiments. This was further confirmed by the fact that when only one enantiomer was derivatized (samples 1 and 13) only one peak was observed.

Fig. 3A and B shows that there were difficulties when a small percentage of one enantiomer had to be determined in a high percentage of the other enantiomer. These problems were more significant when the first eluting enantiomer was present in a high percentage relative to the second enantiomer. The changed elution order between the two methods makes the combination of the methods serviceable for enantiomeric purity determinations.

TABLE II

PERCENTAGES OF (+)- AND (−)-PROMETHAZINE IN SAMPLES 1–13

See Fig. 3A and B.

Sample No.	(+)-Promethazine			(−)-Promethazine		
	Attempted	Analyzed		Attempted	Analyzed	
		Direct	Indirect		Direct	Indirect
1	100	100	100	100	100	100
2	99	100	99.6	99	99.0	99.6
3	90	86.2	90.6	90	89.3	88.0
4	80	76.2	80.5	80	80.8	77.0
5	70	66.7	71.7	70	69.8	65.8
6	60	57.6	61.7	60	60.5	57.6
7	50	48.6	52.9	50	51.4	47.1
8	40	39.5	42.4	40	42.4	38.3
9	30	30.2	34.2	30	33.3	28.3
10	20	19.2	23.0	20	23.8	19.5
11	10	10.7	12.0	10	13.8	9.4
12	1	1.0	0.4	1	0.1	0.4
13	0	0	0	0	0	0

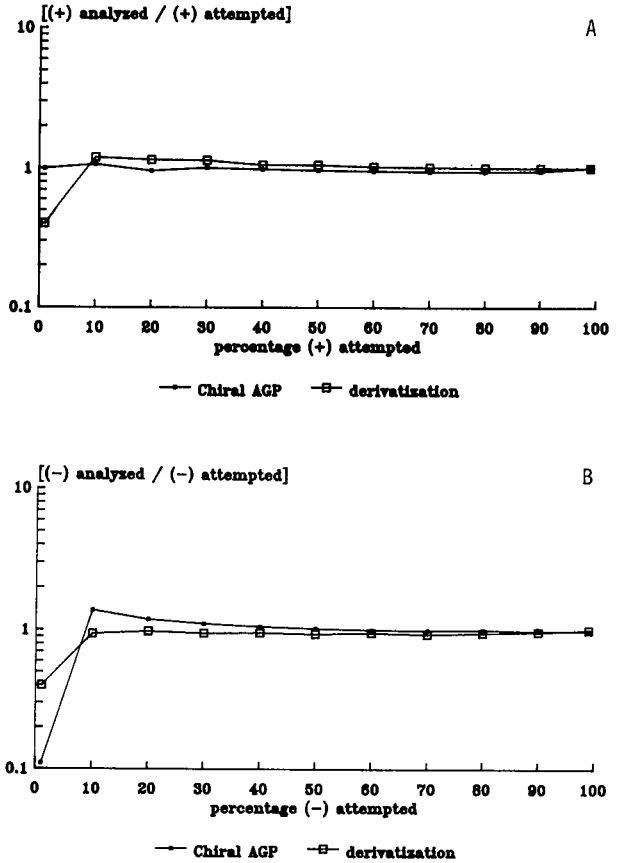

Fig. 3. (A) Percentage (+) attempted divided by the percentage (+) analyzed relative to the percentage (+) attempted in two different separation systems. (B) Percentage (−) attempted divided by the percentage (−) analyzed relative to the percentage (−) attempted in two different separation systems.

TABLE III

RESOLUTION (R) BETWEEN THE DIASTEREOISOMERS OF THE FOUR DERIVATIZED AMINES AND THE LOG CAPACITY FACTORS (LOG k') OF THE FIRST ELUTING DIASTEREOISOMER IN THREE ELUENT COMPOSITIONS

Amine	Eluent A		Eluent B		Eluent C	
	R	log k'	R	log k'	R	log k'
Promethazine	2.0	0.60	2.5	0.97	3.2	1.38
Trimeprazine	1.0	0.81	1.2	1.16	1.4	1.59
Trimipramine	0.0	0.88	>0.1	1.27	0.6	1.73
G-22150	1.1	0.65	1.8	1.02	2.3	1.44

TABLE IV

EFFECTS OF CHANGES IN THE RING STRUCTURE OR THE CHAIN LENGTH OF THE DERIVATIZED AMINE ON RESOLUTION AND LOG k'

Change	Mean R			Mean log k'		
	Eluent A	Eluent B	Eluent C	Eluent A	Eluent B	Eluent C
Phenothiazine	1.5	1.85	2.3	0.705	1.065	1.485
↓	↓	↓	↓	↓	↓	↓
Iminodibenzyl	0.55	0.90	1.45	0.765	1.145	1.585
Effect on R	−0.95	−0.95	−0.85			
Effect on log k'				+0.060	+0.080	+0.100
2-(Dimethylamino)-2-methylethyl	1.55	2.15	2.75	0.625	0.995	1.410
↓	↓	↓	↓	↓	↓	↓
3-(Dimethylamino)-2-methylpropyl	0.50	0.60	1.00	0.845	1.215	1.660
Effect on R	−0.95	−1.55	−1.75			
Effect on log k'				+0.220	+0.220	+0.250

Influences of chemical structure of the tertiary dimethylamines on capacity factors and resolutions of the derivatization products in three eluent compositions in RP-HPLC.

The four amines depicted in Fig. 1 were derivatized with (−)-menthylchloroformate. The influences of the differences in chemical structure between these amines on resolutions and capacity factors were calculated. The results are summarized in Tables III and IV.

Tables III and IV show that the resolution between the diastereoisomers was influenced by the distance between the chiral centres: the shorter the distance the better the resolution. The type of ring structure also had a significant effect on resolution and capacity factor.

It is clear that resolution increased with an increasing amount of acetic acid in the eluent and that the capacity factors increased at the same time, resulting in excessively long retention times at acetic acid concentrations > 15%. The reproducibility of the resolution of diastereoisomers on different columns with the same stationary phase is a common problem. For the promethazine derivatives two batches of Machery-Nagel Nucleosil C_{18} 5 μm and one Merck LiChrospher 100 RP-18 5 μm were tested. No differences between the batches or between the brands were observed.

CONCLUSIONS

During the derivatization of promethazine with (−)-menthylchloroformate no racemization occurred. The chromatographic results of the indirect method showed a higher resolution (R) and plate number (N) than the direct method. The difference in elution order for the promethazine enantiomers between the two methods makes the combination of these two methods very useful in the determination of enantiomeric purity of the promethazine enantiomers.

The resolution of the diastereoisomers in the indirect method was influenced not only by the distance between the chiral centres in the diastereoisomers, but also by the ring structure. The iminodibenzyl derivatives showed higher capacity factors

than the corresponding phenothiazines. On the other hand, the phenothiazine derivatives were better resolved than the corresponding iminodibenzyls.

More acetic acid caused a better resolution. However, the influence on capacity factor was such that higher amounts of acetic acid in the eluent resulted in unacceptable retention times.

The indirect method is simple and fast and therefore a good and cheap alternative to the direct one. The applicability of the derivatization method in biological samples has shown to be promising in our hands.

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CHROMSYMP. 2132

(*S,S*)-Diphenylethylethanediamine derivatives as chiral selectors

II. Gasparrini-type bound chiral stationary phase with high enantioselectivity for naphthylamides[a]

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ABSTRACT

The synthesis of a chiral stationary phase (CSP) containing the 3,5-dinitrobenzoyl derivative of (*S,S*)-1,2-diphenylethanediamine (DPEDA) as a chiral selector by immobilizing the DPEDA derivative onto glycidoxypropyl-modified silica gel and subsequent benzoylation is described. This so-called CSP-II showed high enantioselectivity under normal-phase conditions, in particular for chiral compounds containing a naphthylamide functionality.

INTRODUCTION

In Part I [1], we reported the synthesis of a new chiral stationary phase (CSP-I) derived from (*S,S*)-1,2-diphenylethanediamine (DPEDA) as chiral selector (SO). We presented some preliminary results concerning the optical resolution capability of this CSP-I (see Fig. 1) under normal-phase high-performance liquid chromatographic (HPLC) conditions. It was found that CSP-I effectively resolved a wide range of racemic selectands (SAs), probably owing to intermolecular π-donor–acceptor and hydrogen-bonding interactions. As indicated in Fig. 1, CSP-I provides a 3,5-dinitrophenyl group as π-donor and two different amido functions as hydrogen donor–acceptor groups. In this work we investigated the influence of a second 3,5-dinitrobenzoyl function in a similar (*S,S*)-DPEDA-derived CSP (CSP-II) on the overall

[a] Dedicated to Professor Dr. G. Zigeuner (Karl-Franzens-University of Graz) on the occasion of his 70th birthday.

0021-9673/91/$03.50

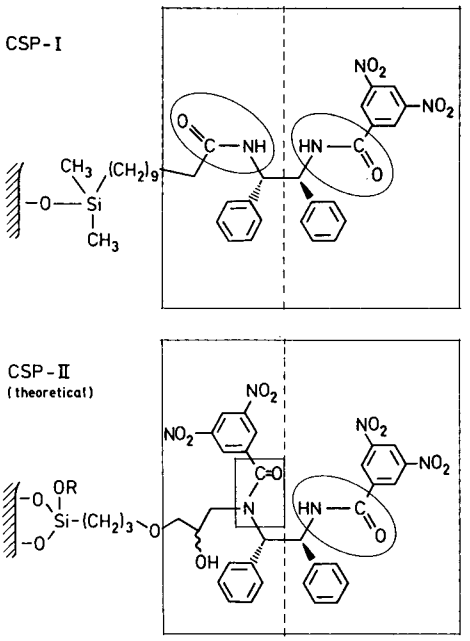


Fig. 1. Structures of CSP-I and CSP-II. The representation of the formulae should assist the visualization of structural elements (*e.g.*, π-acid, hydrogen donor–acceptor, stereogenic centre) responsible for intermolecular interactions with suitable guest molecules. Ellipse = secondary amide; rectangle = tertiary amide; dashed line = quasi-*C*-2 axis.

enantioselectivity. Systematic studies by Pirkle and Pochapsky [2], who evaluated the enantioselectivity of various rationally designed CSPs, have shown that the space filling size, the configuration of the stereogenic centres and hydrogen-donating amido groups must influence the overall selectivity of such modified chiral selectors. Further, the conformation of the immobilized selector molecules is also important for the observed chiral recognition. It should be dependent on the kind and size of the "spacing arm" to the silica surface. Also, as stressed recently by Däppen *et al.* [3], in all discussions of enantioselective chromatographic retention mechanisms it should be mentioned that the observed retention and resolution data are due to the total sum of chiral and non-chiral interactions of the SAs on a given CSP.

The new CSP-II was prepared following a straightforward synthetic route developed by Gasparrini and co-workers [4,5] for *trans*-diaminocyclohexane (DACH). In our particular case we reacted (*S,S*)-DPEDA with a glycidoxypropyl-modified silica gel followed by exhaustive benzoylation with 3,5-dinitrobenzoyl chloride (DNB-Cl) (Fig. 2). Surprisingly, this synthetic variation of the Gasparrini approach led to a mixture of mono- and dibenzoylated chiral selectors, as depicted in Fig. 2 and explained under Experimental. Nevertheless, the new CSP-II was found to have interesting separation capabilities, different from those of CSP-I. We made comparative studies of CSP-I and the much easier and more economically prepared CSP-II under similar chromatographic conditions and with the same set of chiral analytes.

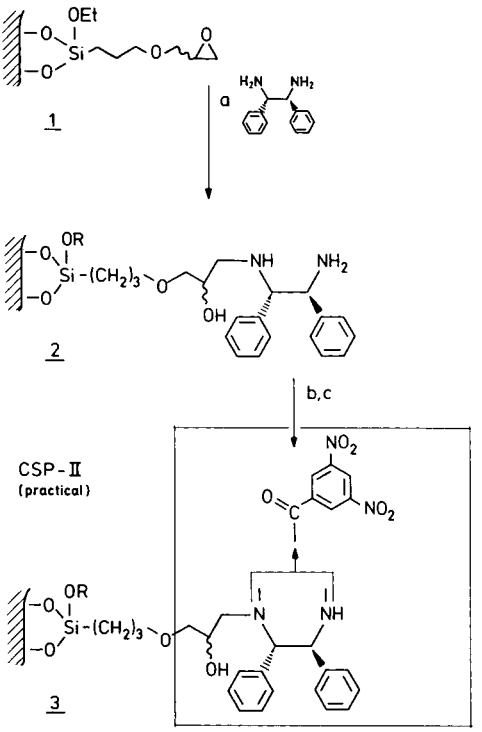


Fig. 2. Reaction scheme for preparing CSP-II. (a) 2 g (*S,S*)-DPEDA, 5.8 g epoxide silica gel **1**, 50 ml ethanol (95%), 20 mg phenol, 11 h gentle stirring and reflux. (b) 2 g **2**, 25 ml methylene chloride, 0.5 ml triethylamine, 0.5 g (4 mol equiv.) DNB-Cl, 20 h gentle stirring, room temperature. (c) Methanol-washed silica gel, 20 ml methanol, 0.5 ml concentrated aqueous ammonia, 3 h, room temperature.

EXPERIMENTAL

Apparatus

Chromatography was performed using a Model 410 HPLC pump, a UVIKON UV–VIS detector and an ANACOMP data control station from Kontron (Zürich, Switzerland). A Rheodyne Model 7125 injector with a 20-μl sample loop was connected to a stainless-steel column (125 × 4 mm I.D.). packed with CSP-II using chloroform–dioxane (3:1) as slurry solvent and *n*-heptane as pressurizing solvent.

Chemicals and reagents

Racemic and optically pure drugs were obtained from different pharmaceutical companies. The derivatives (amides, carbamates, ureas) of the various chiral acids, amines and alcohols (obtained from Aldrich, Steinheim, Germany) were prepared by common methods using the respective acid chlorides or isocyanates. Glycidoxypropyltriethoxysilane and 3,5-dinitrobenzoic acid were obtained from Aldrich and LiChrosorb Si 100 (5 μm), HPLC-grade solvents and other common chemicals used for the synthesis of CSP-II from Merck (Darmstadt, Germany). The remaining compounds were available from previous studies [1].

Synthesis of CSP-II

(*S,S*)-DPEDA was synthesized from benzil and ammonia and resolved optically according to a recently published method [6].

Glycidoxypropylsilica was prepared by gently stirring 10 g of silica gel in a refluxing solution of 3 g of glycidoxypropyltriethoxysilane in 200 ml of toluene that had been saturated with water by ultrasonication. After 10 h the modified silica gel was isolated, washed twice with 150 ml of toluene and diethyl ether and dried at 60°C. Elemental analysis gave C 5.34 and H 1.17% (H values are usually not very representative for such a type of analysis).

According to the reaction scheme depicted and briefly described in Fig. 2, the epoxide of the functionalized silica gel reacts with the large excess of (*S,S*)-DPEDA to give the addition product **2**. Elemental analysis gave C 9.55, H 1.54 and N 0.81%. Based on the nitrogen value, a coverage of 280 μM of covalently bonded (*S,S*)-DPEDA per gram of epoxidized silica gel was calculated. The yield of the addition reaction was about 70%.

Subsequently, **2** was exhaustively acylated with excess of DNB-Cl at 60°C and an appropriate base. As judged by the C,N elemental analysis, the benzoylation yield was nearly the same when triethylamine was replaced with pyridine as solvent and HCl scavenger. In order to cleave the formed DNB ester of the secondary hydroxy group (qualitatively judged by DRIFT spectroscopy) the modified silica gel, extensively washed with methanol, was subsequently treated for 3 h with 0.5 ml of concentrated aqueous ammonia in methanol followed by extensive washing with methanol. The content of cleaved DNB acid was measured by UV absorption of the washing solutions. The elemental analyses of the final, vacuum-dried material 3 were C 11.53, H 1.49 and N 1.64%, and for a second batch (acylation in pyridine followed by ammonia cleavage of the material with 0.1 *M* hydrochloric acid in methanol) C 11.33, H 1.46% and N 1.58%. Based on the nitrogen values found for the immobilized compounds **2** and **3**, we calculated a yield of the bis-(N-3,5-dinitrobenzoyl)-DPEDA product of about 60%. This reaction yield is not unusual for a solid-phase type of reaction within pores of varying pore-size distribution.

The final CSP-II material was slurry-packed (chloroform–dioxane) into a 125 × 4 mm I.D. stainless-steel column.

RESULTS AND DISCUSSION

CSP-I and CSP-II are similar; both contain one 3,5-dinitrobenzoyl group and a secondary amido functionality at the same position (see the right-hand boxed parts of the CSP-I and CSP-II molecules depicted in Fig. 1). This part is also identical with the well known "Pirkle phase" derived from 3,5-dinitrobenzoylphenylglycine [7]. In contrast to the right-hand parts, the left-hand parts of the two CSP molecules vary: CSP-I contains two secondary amido groups, whereas CSP-II, which is actually a mixture of mono- and bis-3,5-dinitrobenzoylamides of the DPEDA–epoxide adduct, as symbolized by the formula CSP-II in Fig. 2, partially contains a tertiary amido group. This has no hydrogen-donating capability.

A further distinction between CSP-I and CSP-II is the existence of a new but racemic secondary hydroxyl group as a third stereogenic centre in the CSP-II molecule. Consequently, CSP-II is inhomogeneous in terms of its molecular constitution.

including the fact that it is a mixture of diastereoisomers. However, Gasparrini and co-workers' per-3,5-dinitrobenzoylated 1,2-diaminocyclohexane (DACH) [4,5], which could suffer from similar inhomogenities, proved to be highly stereoselective and reproducible in production.

The new CSP-II proved to be enantioselective for a representative range of chiral analytes; the results are summarized in Table I. The chiral acids, amines and alcohols were derivatized to amides, ureas and carbamates, respectively, thus incorporating polar functional groups which might participate as intermolecular interaction sites. As CSP-II has 3,5-dinitrobenzoyl groups serving as π-acids, they should interact with π-bases depending on their basicity. Together with hydrogen-bonding, hydrogen-donating or dipole–dipole stacking sites, paired with the spatial arrangements of the two stereogenic centres (1*S*,2*S*-position), CSP-II, and also CSP-I, display enantioselective multiple contact areas accessible to interaction via complementary binding forces of the chiral analytes.

This is a very generalized statement for explaining particular enantioselectivities. Although CSP-II shows good enantioselectivity it does not seem reasonable at this point to postulate and discuss distinct binding models, as CSP-II is not homogeneous in terms of its chemical constituents (it is also a mixture of diastereoisomers). As can be seen from the data in Table I, various enantiomers and particularly those containing a 1-naphthyl group, are well resolvable on CSP-II. Further, the observed enantioselectivity values (α) for this group of compounds are, with one exception, higher than the corresponding values acquired on CSP-I under comparable mobile phase conditions.

The mean retention time of the compared and resolved pairs was between 10% and 40% lower on CSP-I than on CSP-II, although the two CSPs have similar loadings on a molecular basis (between 280 and 300 μmol of selector per gram of silica gel). Therefore, the degree of benzoylation is higher on CSP-II than on CSP-I, which might influence the retention characteristics of compounds containing strong π-base groups.

However, this trend seems particularly true for well defined and simple compounds having a naphthyl group as a substituent either at the stereogenic centre or at another position of the derivatized chiral analyte.

The more complex the chiral compounds (SAs) are structured, *e.g.*, the oxazolidin-2-ones of beta-blockers but also benzodiazepines, the less (spatially) defined the intermolecular SO–SA interactions might be.

In this context, one should mention that the observed differences in the retentions of stereoisomers on a given CSP are due to a "mixed mode" binding contribution of chiral and non-chiral interactions between the SO and SA molecules [3]. Therefore, by comparing CSP-I with CSP-II, the differences of the spacer parts between the chiral DPEDA molecule part and the silica gel surface should not be ignored (see Fig. 1). The spacer length, for instance, might influence the overall retention and enantioselectivity characteristics to a certain extent. In this respect, it is interesting that the above-mentioned benzodiazepines (Table I, lormetazepam) are significantly less resolved [1] and oxazolidine-2-ones (not shown in Table I) are poorly resolved on CSP-II. On the other hand, the separation capabilities for sulphoxides are not predictable. Simple benzyl phenyl sulphoxide is resolved only on CSP-I, whereas more complicated structures are separated solely with the higher benzoylated CSP-II.

From the present observations and by studying the extensive literature on

TABLE I

ENANTIOSEPARATION OF REPRESENTATIVE ANALYTES ON CSP-II

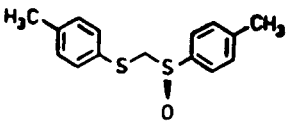
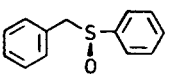
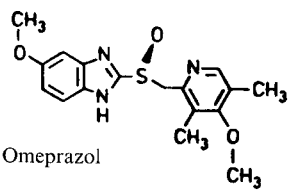
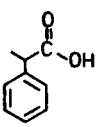
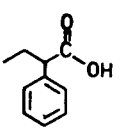
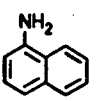
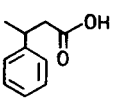
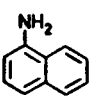
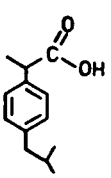
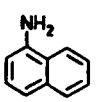
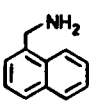
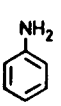
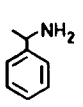
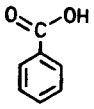
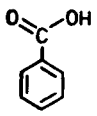
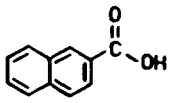
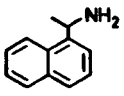
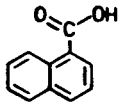
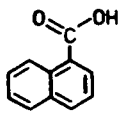
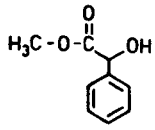
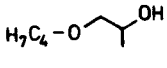
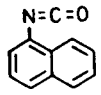
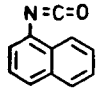
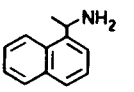
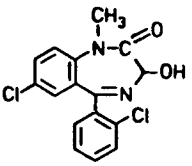
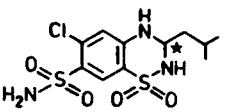
Compound	Derivatization reagent	Mobile phase[a]	k'_1[b]	α[c]	R_s[d]	Elution order	α on CSP-I[e]
Sulphoxides							
		A	3.72	1.27	1.51		1.0
		B	1.79	1.08	0.81		1.0
		A	1.33	1.0	–		1.16
		B	2.12	1.0	–		1.22
 Omeprazol		A	6.33	1.0	–		1.0
		B	7.14	1.12	0.50		1.0
Acids as amides							
		A	2.50	1.66	3.73		1.44
		B	1.33	1.33	2.33		–
		A	2.0	1.65	3.95		1.39
		B	1.0	1.33	2.33		–
		A	2.33	1.09	0.50		1.11
		B	1.72	1.0	–		–
		A	1.72	1.97	4.42	*S*>*R*	1.36
		B	1.21	1.32	2.57	*S*>*R*	–
		A	1.33	1.66	3.11	*S*>*R*	1.34
		B	1.15	1.18	1.37	*S*>*R*	1.0
		A	2.03	1.09	0.58		1.0

TABLE I *(continued)*

Compound	Derivatization reagent	Mobile phase[a]	k'_1[b]	α[c]	R_s[d]	Elution order	α on CSP-I[e]
Amines as amides							
		A	1.08	1.20	1.15	*R*>*S*	1.18
		B	1.24	1.21	1.74	*R*>*S*	–
		A	3.05	1.65	3.0	*R*>*S*	1.43
		B	2.23	1.47	3.60	*R*>*S*	–
		A	2.0	1.30	2.13	*R*>*S*	1.18
		B	1.66	1.18	1.06	*R*>*S*	–
		A	2.14	2.52	4.90	*R*>*S*	1.72
		B	1.59	1.88	4.21	*R*>*S*	1.09
		A	8.27	3.40	4.78		1.28
		B	3.35	2.26	4.51		1.0
Alcohols as carbamates							
		A	1.72	1.39	2.37		1.37
		B	0.65	1.27	1.17		1.0
		A	2.94	1.07	0.40		1.0
		B	0.94	1.0	–		–
		A	1.53	1.15	1.18		–
		B	0.53	1.01	–		–
Amines as ureas							
		B	4.0	1.32	2.71	*R*>*S*	–
		A	2.44	1.17	1.26		1.25
		B	3.11	1.12	1.18		–

(Continued on p. 380)

TABLE I *(continued)*

Compound	Derivatization reagent	Mobile phase[a]	k'_1[b]	α[c]	R_s[d]	Elution order	α on CSP-I[e]
		A	2.22	1.95	4.06	$R>S$	1.26
		B	2.33	1.44	3.12	$R>S$	–
Miscellaneous							
		A	5.88	1.15	0.67		1.37
		B	3.70	1.10	1.23		1.29
Lormetazepam							
Butizide							
		C	4.63	1.11	0.56		1.24

[a] Mobile phases (%, v/v): (A) *n*-heptane–2-propanol–diethylamine (DEA) (70:30:0.1); (B) *n*-heptane–dioxane–DEA (70:30:0.1); (C) *n*-heptane–dioxane (50:50); flow-rate 0.9 ml/min; the column was thermostated at 20°C.

[b] Capacity factor: $k' = (t_r - t_0)/t_0$.

[c] Selectivity: $\alpha = k'_2/k'_1$.

[d] Resolution: $R_s = (\alpha-1)/4\alpha \cdot k'_2/(k'_2+1) \cdot \sqrt{N_2}$.

[e] Most of these data have been published previously [1]; the mobile phases used on CSP-II and CSP-I are identical.

chromatographic enantioseparations [8,9], it is clear that the total structure, the configuration of the stereogenic centres and subsequently the conformation of chiral selector molecules are of great importance for chiral recognition. Large parts of the selected molecules (CSP-I and CSP-II) are identical (the substituents at the stereogenic centres, their configurations and the amides as functional groups), as shown in idealistic form in Fig. 1. Realistically we have to deal with a less defined CSP-II structure expressed by the formula 3 in Fig. 2. The new but racemic hydroxyl group in CSP-II, however, seems to influence considerably its overall stereoselectivity compared with CSP-I, although we never observed a reversal of elution order.

Assuming ideal and homogeneous situations, (*S,S*)-DPEDA is a molecule with *C*-2 symmetry and the acyl derivatives thereof (see the molecule structures of CSP-I and CSP-II in Fig. 1) still have a quasi-*C*-2 symmetry, as indicated by the dotted line. With the aid of this line it also becomes clear that CSP-I contains two relatively similar secondary amido functions (as indicated by the elliptical lines in Fig. 1), whereas the main proportion of CSP-II contains a secondary and a tertiary amido function.

As mentioned above, the right-hand part of the quasi-*C*-2 symmetrical molecule

is equivalent to the chiral 3,5-dinitrobenzoylphenylglycine molecule part of the corresponding CSP introduced by Pirkle *et al.* [7]. Consequently, and assuming a three-point interaction model for chiral recognition, Pirkle *et al.*'s and our two CSPs should resolve selected enantiomers in a similar way. This could be confirmed for, e.g., the simple 1-naphthoyl derivative of (*R,S*)-phenylethylamine which was separated in the same elution order.

In the present (1*S*,2*S*)-DPEDA-type CSPs there are two adjacent stereogenic centres with the same configuration and which are substituted with similar amide functionalities. However, at this point it is not clear whether the "doubling" of similar chiral molecule parts within one chiral molecule will act synergistically in terms of "additively" enlarging the overall enantioselectivity. As we could see from various stick and ball and spacefill models, this seems unlikely owing to predominant conformational changes. Thus, "stereogenic contact areas" get exposed, leading to hardly predictable enantioselectivity models.

CSP-I and CSP-II are starting points for studying more systematically quasi-*C*-2 symmetrical but similar multifunctionalized chiral selector molecules and the contribution of stereoselective increments. This is of considerable interest to us and is the subject of ongoing research in the field of chiral recognition in liquid chromatography.

Consequently, a logical extension of CSP-II is to synthesize a well defined mono-N-3,5-dinitrobenzoyl-(*S,S*)-DPEDA derived CSP. Results on these aspects will be published elsewhere [10].

In conclusion, the described and easily prepared CSP-II is, depending on the method of synthesis, not fully homogeneous in terms of the molecular structure of the chiral selector. However, the synthesis is reproducible. The column efficiency of the CSP-II packing is good and comparable to those of typical π-acid–π-base "Pirkle-type" CSPs. Depending on the structure of the chiral analytes, reduced plate heights (h) between 3 and 8 were observed, leading to plate numbers (N) between 20 000 and 65 000 per metre.

CSP-II proved practically useful and showed higher enantioselectivity towards chiral analytes containing a naphthyl group than CSP-I [1]. CSP-II is chemically and thermally stable; after long-term usage (2000 column volumes of a non-aqueous mobile phase were continuously pumped through the column at 60°C), the retention and the enantioselectivity decreased by only about 5%.

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Reversed-phase and chiral high-performance liquid chromatographic assay of bupivacaine and its enantiomers in clinical samples after continuous extraplural infusion

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ABSTRACT

A previously unreported coupled achiral–chiral high-performance liquid chromatographic method has been developed to assay the levels of bupivacaine and its enantiomers in plasma samples, after the local anaesthetic had been given as a continuous extrapleural intercostal nerve block for 4 days, to relieve postoperative pain following thoracotomy. The method has been used to determine maximum, individual enantiomer and steady state levels in conjunction with an assessment of whether accumulation of bupivacaine occurs. An off-line extraction sample preparation is involved before determination of the "total" levels and final sample clean-up on a cyanopropyl silica column prior to "heart cutting" of the bupivacaine peak to a Chiral-AGP column for assay of the enantiomeric ratio. For an initial 5 patient number the mean maximum level was 5.43 μg/ml against a reported toxic level of around 5 μg/ml, which was reached in 72 h and the *S*-enantiomer gave slightly increased concentrations over the *R*-enantiomer for which there is some evidence of higher toxicity.

INTRODUCTION

The local anaesthetic, bupivacaine has a relatively long duration (active up to 8 h) and can be utilised for nerve blockade in extended term surgery. One area where the anaesthetic has proved particularly useful is in thorocotomy. Postoperatively, however, thorocotomy can produce a high incidence of severe pain and pulmonary complications. Patient comfort after this major surgery is regularly carried out by employing systematic opiates. But this form of pain relief is known in some cases to be inadequate [1] and added to this central respiratory depression is problematic. Numerous alternatives have been suggested and these include cryonalagesia, intercostal and epidural nerve blocks.

Recently Sabanathan *et al.* [2] have demonstrated that continuous infusion of bupivacaine over a 24-h period by an indwelling epidural catheter placed extrapleurally at thoracotomy can be beneficial to control pain. Subsequently this study has

0021-9673/91/$03.50

been enlarged and the period of induction of bupivacaine by this mode has been extended to 5 days. As a result decreased pain and improved lung function have been illustrated when comparisons have been made against conventional analgesia [3]. Improved coughing ability and restoration of normal breathing pattern have lead to improved recovery times and early patient mobility.

However, over the 5 day period of infusion, the dosage of bupivacaine is necessarily high, but in observation tests no clinical toxic reactions, which are apparent as myocardial depression and convulsions, were found over a large patient population [4].

Nevertheless it was considered important to establish the maximum levels of bupivacaine in patient samples during and after infusion. In order to carry this out, subsequent to liquid extraction from a plasma biological fluid, a reversed-phase high-performance liquid chromatography (HPLC) method was developed. A number of previous methods have utilised alkyl-bonded phases for determination of bupivacaine levels in serum [5,6]. The assay is however complicated by the presence of a chiral centre in bupivacaine and the reported differences in toxicity of the *R* enantiomer over the *S* enantiomer [7]. In addition the *S* enantiomer is considered to have a longer duration of action which manifests from the differences in absorption into the tissues [8]. The developed HPLC method therefore includes a conventional assay for the total bupivacaine and an resolution on a chiral stationary phase for the enantiomeric ratio, with HPLC column coupling through switching valves.

EXPERIMENTAL

Patient samples

Plasma samples were obtained from 10 consenting adults who were undergoing elective thoracotomy and were subjected to continuous extraplural infusion of bupivacaine over 4 days. The patients had no infection, lung resection, grossly obese or loosing more than 500 ml of blood peroperatively. Infusion rates varied from 5–10 ml/h of a 5-mg/ml bupivacaine solution according to body weight. Samples were taken through an indwelling central venous catheter at 0, 10, 20, 30, 45, 60, 80, 120, 180, 210, 300, 360, 480 and 24, 48, 72, 96, 120 h. They were subject to centrifugation without additives to give the plasma portion which was stored at −40°C prior to analysis.

Sample preparation

An aliquot of plasma (1 ml) was shaken with 2 *M* sodium hydroxide (100 μl) and hexane (7 ml) in a separating funnel for 10 min. From the upper hexane layer 4 ml was transfered to a centrifuge tube (15 ml) and the solvent evaporated under nitrogen. The residue was redissolved in 200 μl of mobile phase (mobile phase 1) and 50 μl was injected onto the cyanopropyl HPLC column.

HPLC equipment and methods

The HPLC instrumentation for the coupled column experiments consisted of two dual reciprocating pumps, a variable-wavelength detector, two high-pressure switching valves and a low-pressure valve (Fig. 1).

For the reversed-phase separation (total bupivacaine) and LDC/Milton Roy

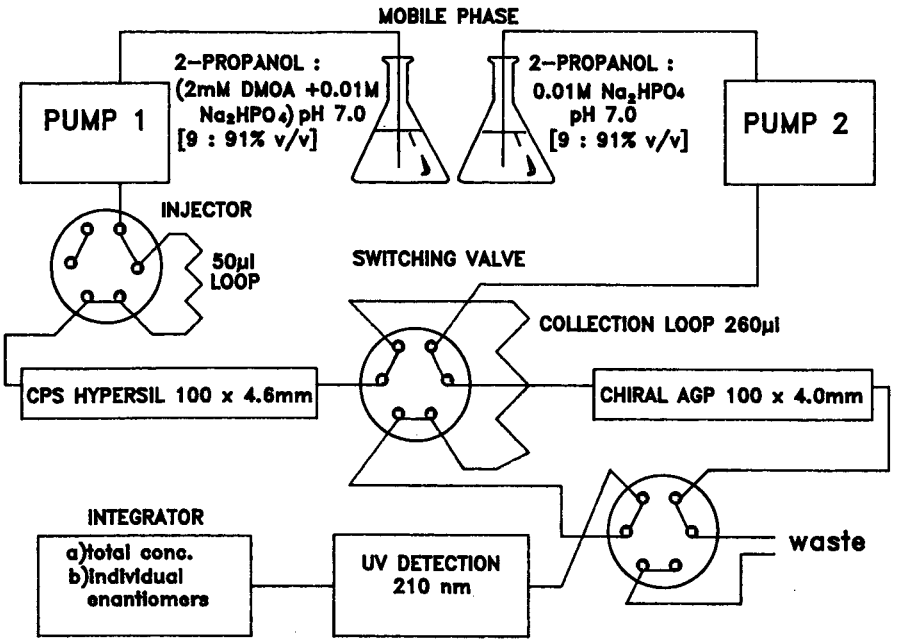

Fig. 1. Configuration of the coupled system, where after initial off-line sample preparation the CPS-column is used for final clean-up and for the determination of the "total" bupivacaine. The chromatogram is then heart-cut to a 260-µl loop for injection onto the Chiral-AGP column in order to obtain the enantiomeric ratios for bupivacaine. Apart from the slight differences in mobile phase compositions between the two columns, there are differences in flow-rate (CPS, 2 ml/min; AGP, 0.9 ml/min).

constametrics 3000 pump was used (LDC/Milton Roy, FL, U.S.A.) with a Rheodyne injection valve fitted with a 50-µl loop (Model 7125, Rheodyne, CA, U.S.A.). The detector for the total levels and individual enantiomeric ratios was an LDC/Milton Roy Spectromonitor 3000 variable-wavelength detector fitted with a 14-µl flow cell. The switching valve between columns and detector was a low-pressure Omnifit Model 1105 valve (Omnifit, Cambridge, U.K.).

The reversed-phase HPLC column was a 100 × 4.6 mm I.D. stainless-steel column packed with 5 µm Cyanopropyl (CPS) Hypersil (Chromex, Cheshire, U.K.). The mobile phase was 2-propanol–0.01 *M* disodium hydrogen orthophosphate + 2 m*M* dimethyl-*n*-octylamine (9:91, v/v) (pH 7.0) (mobile phase 1). The flow-rate was 2 ml/min and detection wavelength 210 nm,.

The pump for the chiral stationary phase (CSP) method was an LKB Model 2150 (LKB Produkter, Bromma, Sweden) fitted with a Rheodyne Model 7000 switching valve and the integrator for both systems was a Hewlett Packard Model HP 3396A (Hewlett Packard, Waldbronn, Germany).

The CSP was a 100 × 4.0 mm I.D. stainless-steel column packed with 5 µm Chiral-AGP (α_1-acid glycoprotein) (ChromTech AB, Norsborg, Sweden). A mobile phase of 2-propanol–0.01 *M* disodium hydrogen orthophosphate (9:91, v/v), (pH 7.0) was used, with a flow-rate of 0.9 ml/min and a detection wavelength of 210 nm.

The mobile phase components: disodium hydrogen orthophosphate was Analar grade and obtained from BDH (BDH, Dorset, U.K.), the dimethyl-*n*-octylamine from Lancaster Synthesis (Lancaster Synthesis, Lancashire, U.K.) and 2-propanol and hexane were obtained as HPLC grade (Rathburn Chemicals, Perthshire, U.K.).

Bupivacaine hydrochloride injection (0.5%) was as a 10-ml ampoule (5 mg/ml) (Astra Pharmaceuticals, Herts, U.K.).

RESULTS AND DISCUSSION

The benefits of column switching in multicolumn HPLC for bioanalysis has been apparent for a number of years [9]. Biological sample preparation, analyte concentration through peak compression and selective component column transfer have all been actively practised. Among the most recent developments in this area are the applications in multidimensional chromatography for chiral drug and metabolite separations in bioanalysis, through the coupling of achiral–chiral columns [10]. Primarily the approach has been to directly inject biological samples for separation and sample clean-up through an initial reversed-phase column [11], then switching the chromatographic peak(s) of interest to the second chiral stationary phase for enantiomeric resolution. This initial step has been successfully illustrated on a number of efficient serum and urine fractionations by Wainer where a Pinkerton internal surface reversed-phase column has been utilised [12].

Although the use of such a column is advantageous, particularly where automation is to be involved, in the development of the bupivacaine HPLC method the cost was considered prohibative when this column's main purpose was in scaveging. Therefore a conventional reversed-phase method was investigated and a number of alkyl-bonded phases were examined. Unfortunately from this work it was not found possible to optimise the packing–mobile phase combination to allow efficient cleanup of the precolumn after every injection and occasional endogenous peak breakthrough occured on random multiple biological sample injections.

As it was intended that the second column would involve a Chiral-AGP packing, which has been shown by Hermansson [13] to be effective in resolving a racemic mixture of bupivacaine enantiomers, care was required in avoiding poisoning of the phase with endogenous material.

As a result an off-line liquid–liquid extraction sample preparation was developed with hexane, which allowed about a 3 times preconcentration, with final clean-up and separation on the achiral column with a 94% (w/w) recovery. For this a column packing of cyanopropyl-bonded silica was found to give both the best separation from the residual proteinatious background and good chromatographic peak shape for bupivacaine as the single "total" level peak. In achieving the optimum separation there was close agreement between the best mobile phase for the achiral and chiral stationary phases. The difference related to the use of ion-pairing mode in the CPS-column, with dimethyl-*n*-octylamine (2 m*M*) and the flow-rate of the mobile phase (CPS, 2.0 ml/min and AGP, 0.9 ml/min). But all other proportions including pH were the same.

The method thus consisted of sample preparation off-line and determination of the "total" bupivacaine levels for the patient samples on the CPS-packing through duplicate injections of 50 μl of prepared sample in mobile phase 1 (Fig. 2). The first

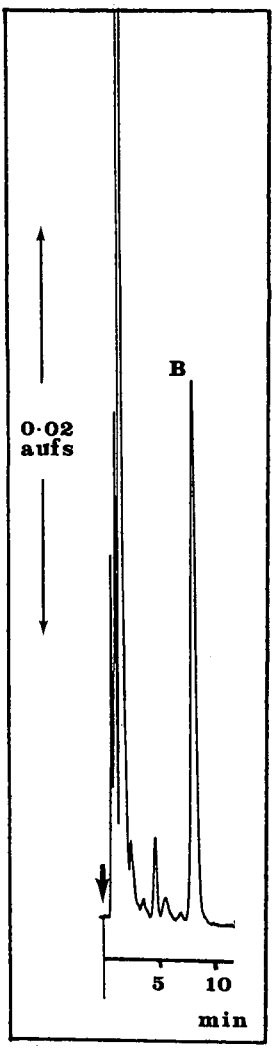

Fig. 2. Determination of the "total" bupivacaine levels on the CPS-Hypersil column, in a 72-h patient sample, after continuous infusion of bupivacaine, (bupivacaine 5.72 μg/ml, 50 μl injected) (for chromatographic conditions, see text).

switching valve allowed the chromatographic profile to be passed directly to the UV detector. Duplicate injections were then carried out (50 μl) where the bupivacaine peak was "heart-cut" from the first separation through manual valve positioning and passed through the switching valve into the 260-μl injection loop. With the manual controlled timing of the Rheodyne switching valve about 50% of the bupivacaine peak was transferred through injection onto the Chiral-AGP column. Baseline resolution of the enantiomorphs was achieved throughout the study with no interference apparent from background peaks as illustrated in Fig. 3 for a 72-h plasma sample.

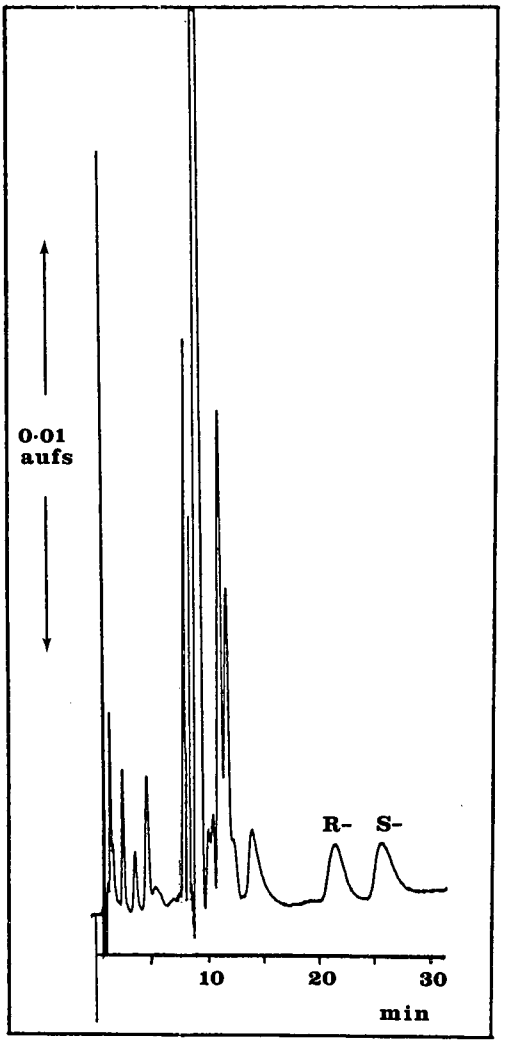

Fig. 3. Determination of the enantiomeric ratios from the chromatogram in Fig. 2 by heart cutting and transfering about 50% (v/v) of the bupivacaine peak to the 260-μl loop for injection and resolution of the *R* and *S* enantiomers on the Chiral-AGP column. The ratios are *R*–*S*, 45.7:54.3% (w/w) (for chromatographic conditions: see text).

Baseline disturbance is however observed in the chromatographic profile at 7–13 min which is considered to be caused by a combination of the 260-μl volume of mobile phase 1 containing dimethyl-*n*-octylamine present on the column and pressure differences associated with injection of this volume. Fortunately these disturbance peaks do not interfere with the resolution of the *R* and *S* enantiomers.

On the CPS column linearity of chromatographic response was determined over two concentration ranges for aqueous bupivacaine standards, as at the outset the

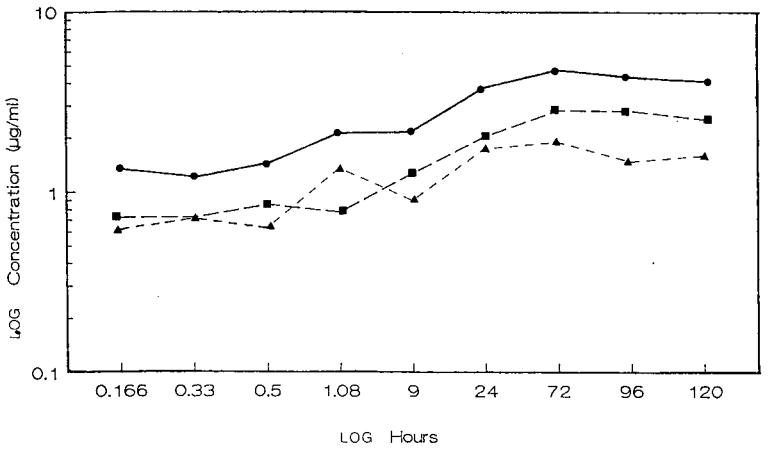

Fig. 4. The plasma concentration against time curve for a 10-patient mean over a 120-h period with a 96-h continuous infusion of bupivacaine. The "total" level (●) was determined on the CPS-Hypersil column and is indicated by where the maximum value (48 h) is at 5.43 µg/ml of bupivacaine. The mean value of the *R* and *S* enantiomers which were resolved on the AGP-column after heart-cutting from the reversed-phase CPS-column, illustrates the slight increase in the *S* enantiomer (■) over the *R* enantiomer (▲) from the original racemic pharmaceutical preparation (for chromatographic conditions, see text).

maximum levels of bupivacaine that the patient samples would reach was not apparent. However, as indicated no clinically toxic effects were shown in the patients and it was therefore expected that the bupivacaine levels would lie around the suggested toxic point of 5 µg/ml [7]. For the concentration range of 1–20 µg/ml of aqueous standards the regression equation was $y = 8.14x + 1.06$ ($R = 0.999$, $n = 12$). Repeatability (R.S.D.) for a standard at 10 µg/ml was 0.58% ($n = 8$). With overlapping higher concentration range 5.02–50.2 µg/ml; again linearity was given and the equation was $y = 2.72x + 1.27$ ($R = 0.999$, $n = 7$). In addition to aqueous standards, spiked standards were run from 5 to 40 µg/ml (spiked in pooled serum). The liquid–liquid extraction was completed as above and the regression equation was $y = 56.34x + 14.29$ ($R = 0.996$, $n = 8$). For the AGP-column linearity was achieved for the *R* isomer with $y = 0.45x + 0.22$ ($R = 0.998$, $n = 5$) and for the *S* isomer, $y = 0.35x + 0.17$ ($R = 0.976$, $n = 5$) for the range 2.5–25 µg/ml. In these assays, for the CPS-column the limit of detection (measured as twice baseline noise) was 13 ng on column for a 50-µl injection.

On the patient samples the plasma concentrations against time curve for a mean of 10 patient samples is shown on Fig. 4. The time to peak plasma concentration was 72 h and the level was 5.43 µg/ml with steady state being reached. There were no suggestions of accumulation of bupivacaine over the 96-h of infusion. After this period there is a slight fall in the mean plasma levels which indicates acceptable clearance from the body. The enantiomeric ratios suggest no significant difference between the enantiomers over the term of study, although the *S* enantiomer is present at a slightly higher level than the *R* enantiomer after 50 h, which is important in view of the reported increase in toxicity from the *R* enantiomer [7]. From these results it is possible to calculate the requisite pharmacokinetic profiles of apparent volume of

distribution, terminal half life and area under the plasma concentration time curve. This will be presented along with full patient data (10-patient sample) in a future report.

CONCLUSIONS

The continuous infusion of bupivacaine has been shown to have considerable benefits for therapy in chronic pain, without the effects of noticable toxic side effects. The analytical results obtained here, compliment these observations and indicate that accumulation of bupivacaine in the body is not problematic. The method developed is easily carried out, although more automation would have been useful in reducing the analyst involvement in sample preparation and switching valve operation. These points however may be considered in future studies of neonate samples and in adult samples through drug combination studies.

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CHROMSYMP. 2170

Liquid chromatographic separation of the enantiomers of metoprolol and its α-hydroxy metabolite on Chiralcel OD for determination in plasma and urine

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ABSTRACT

The two enantiomers of metoprolol and the four enantiomeric forms of α-hydroxymetoprolol were separated by liquid chromatography on a Chiralcel OD column containing a cellulose tris(3,5-dimethylphenylcarbamate) chiral stationary phase. The column efficiency was strongly dependent on the flow-rate and the enantioselectivity was influenced by temperature. Of utmost importance for the chiral separation was the water content of the mobile organic phase. The separation system was used for the separation and determination of the enantiomers in plasma and urine samples. The metoprolol enantiomers could be determined by fluorescence down to 10 nmol/l of each in plasma with a relative standard deviation of less than 15%.

INTRODUCTION

In recent years there has been an increased interest in chiral drugs with regard to pharmacokinetics and pharmacodynamics of the separate enantiomers. The development of new tools for chiral separation, mainly by liquid chromatography, has contributed greatly to the possibility of such studies [1].

Metoprolol, a β-adrenoceptor blocking drug, is one of numerous examples of drugs administered as a racemate, where the two antipodes do not have identical pharmacological properties. We have studied the separation of the enantiomers by liquid chromatography on an α_1-acid glycoprotein chiral stationary phase, Chiral AGP [2], which has found wide applications in drug separations. We have also presented an enantioselective assay for metoprolol in plasma samples at levels down to 2 nmol/l [3].

A family of chiral stationary phases of cellulose triphenylcarbamate derivatives on silica gel has attracted considerable interest during the last few years [4–6]. Recently the separation and determination of metoprolol enantiomers were demonstrated

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using Chiralcel OD [7–9], a stationary phase of cellulose tris(3,5-dimethylphenylcarbamate) polymer coated on macroporous silica, from Daicel. We also examined this phase, not only for separating metoprolol isomers but also for the separation of the isomers of the α-hydroxy metabolite in human urine and in dog plasma. There are two asymmetric carbons in this metabolite, making four isomers to be separated. The aim of this study was to optimize the separation conditions. We examined the influence of flow-rate, temperature and mobile phase water content on column efficiency and selectivity. It was found essential to control the water content of the organic mobile phase as it had a significant influence on the enantioselectivity of the Chiralcel OD phase.

EXPERIMENTAL

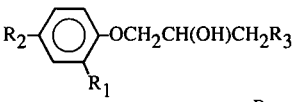
Chemicals

Metoprolol as tartrate, α-hydroxymetoprolol as *p*-hydroxybensoate, (*S*)-alprenolol as chloride, (*S*)-metoprolol as sorbate and H 93/82 (a diol) (Fig. 1) were obtained from the Department of Organic Chemistry, Hässle (Mölndal, Sweden). Hexane and dichloromethane of high-performance liquid chromatographic (HPLC) grade were from Rathburn (Walkerburn, U.K.), diethyl ether of pro analysi grade from May & Baker (Dagenham, U.K.), diethylamine from Fluka (Buchs, Switzerland), 1-propanol and buffer substances from E. Merck (Darmstadt, Germany) and water from a Milli-Q system (Millipore, Molsheim, France).

Instrumentation and conditions

The liquid chromatograph consisted of an LKB (Bromma, Sweden) Model 2150 pump, a WISP 710 B autosampler (Waters Assoc., Milford, MA, U.S.A.) and a Model 821 FP fluorescence detector (Jasco, Tokyo, Japan) operated at 272 nm (excitation) and 306 nm (emission). A laboratory-built thermostat controlled the temperature of the column. The chromatograms were recorded on an SP4400 integrator (Spectra-Physics, San José, CA, U.S.A.). A Metrohm (Herisau, Switzerland) 684 KF coulometer was used to measure the water content of the mobile phase.

The analytical column (250 × 4.6 mm I.D.) was a Chiralcel OD from Daicel (Tokyo, Japan) which was used between 20 and 40°C with flow-rates of 0.25–1.75 ml/min. The mobile phase contained 0.1% of diethylamine, 200–1500 mg/l of water, 4–10% of 1-propanol and hexane.



		R_1	R_2	R_3
I	Metoprolol	H	$CH_2CH_2OCH_3$	$NHCH(CH_3)_2$
II	S–Alprenolol	$CH_2CH{=}CH_2$	H	$NHCH(CH_3)_2$
III	α–OH–metoprolol	H	$CH(OH)CH_2OCH_3$	$NHCH(CH_3)_2$
IV	H 93/82 (diol)	H	$CH_2CH_2OCH_3$	OH

Fig. 1. Structures of metoprolol, metabolites and the internal standard.

Calibration and test solutions

Standard solutions of metoprolol, (*S*)-metoprolol, α-hydroxymetoprolol and (*S*)-alprenolol were made up in 0.01 *M* hydrochloric acid at concentrations of 10, 10, 75 and 20 μmol/l, respectively. Test solutions in the organic mobile phase were prepared by extraction with diethyl ether–dichloromethane (4:1) from alkalinized reference solutions. After phase separation (freezing of aqueous phase) and evaporation under nitrogen the amines in base form were dissolved in the mobile phase.

Analytical procedure

A 1-ml volume of human or dog plasma or urine is mixed with 0.050 ml of internal standard solution [20 μmol/l (*S*)-alprenolol in 0.01 *M* hydrochloric acid] and 0.10 ml of 1 *M* sodium hydroxide solution and the mixture is extracted with 5 ml of diethyl ether by shaking for 10 min. After centrifugation for 5 min the organic phase is evaporated under nitrogen and the residue is dissolved in 0.250 ml of mobile phase, 0.050 ml of which is injected onto the chromatographic column. When α-hydroxymetoprolol is determined, 0.5 g of sodium chloride is added prior to extraction with diethyl ether–dichloromethane (4:1) [10].

Reference samples for calibration are included in the daily series of analyses. A 0.100-ml volume of a standard solution, 2 μmol/l in 0.01 *M* hydrochloric acid, is added to 1 ml of blank plasma in five replicates which, together with one blank plasma sample, are processed in parallel with authentic samples and with daily quality control samples.

Calculations

Peak-height ratios between the analytes and the internal standard, (*S*)-alprenolol, are calculated for each chromatogram by an electronic integrator, which uses the median value of the calibration samples to estimate plasma concentrations of the authentic samples. Full calibration graphs in the range 10–1000 nmol/l are determined at intervals of 1 month during analysis periods.

The parameters used in the evaluation of the column and separation efficiency are the plate number, N, the capacity factor, k', and the separation factor, α:

$$N = 5.54(t_R/W_{1/2})^2$$
$$k' = (t_R - t_o)/t_o$$
$$\alpha = k'_S/k'_R$$

where $W_{1/2}$ = width at peak half-height in minutes, t_R = retention time in minutes and t_o = retention time of injected toluene, which was assumed to be non-retained.

RESULTS AND DISCUSSION

The work-up procedure for metoprolol in plasma was similar to that used in two previous studies [3,10]. The extract from plasma in diethyl ether or diethyl ether–dichloromethane was concentrated by evaporation prior to dissolution in the mobile phase and injection. The two metoprolol enantiomers were separated from each other and from the internal standard, (*S*)-alprenolol, which was eluted in between. Representative chromatograms from blank plasma, and from an authentic human plasma

sample are shown in Fig. 2. The metoprolol antipodes are well isolated from plasma components and, despite the relatively simple work-up [7–9], the measurement of small amounts is possible.

The presence of diethylamine in the mobile phase was needed to permit an acceptable chromatographic performance for metoprolol and other amines. The column efficiency, *i.e.*, the plate number, was strongly dependent on the flow-rate (0.25–1.75 ml/min), but temperature (20–40°C) had little influence. The capacity factors decreased with increase in temperature, more rapidly for the later eluting *S* form than for the *R* form, and the separation factor α decreased.

The water content of the organic mobile phase was not discussed in previous papers [7–9] but is of great importance for the chiral separation, as shown in Fig. 3. On going from 200 to 1400 mg/l of water the retention of the *R* enantiomer was only slightly affected whereas the k' value of the *S* form was almost halved and consequently the separation factor, α, decreased from 2.4 to 1.4. The water content of the organic solvents constituting the mobile phase, in particular the alcohol, may vary considerably and in certain instances differences in air humidity are probably of significance.

Overall we found it important to control the water content of the mobile phase in order to ensure a reproducible chiral separation. This was obvious from the sep-

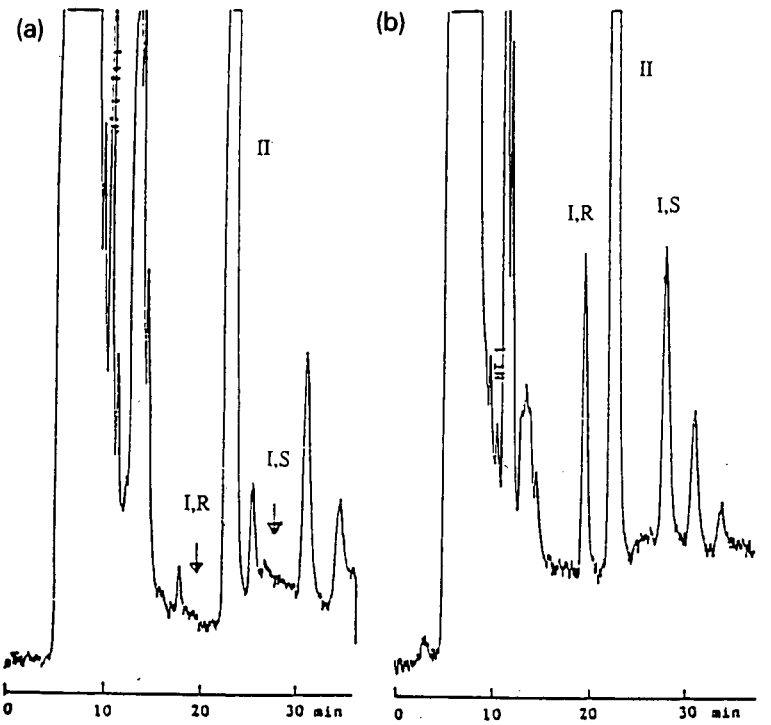


Fig. 2. Separation of metoprolol (I) enantiomers in human plasma. Mobile phase: 4% 1-propanol, 0.1% diethylamine and 1500 mg/l water in hexane; flow-rate, 0.5 ml/min; temperature, 35°C. Integrator attenuation: 8. (a) Blank plasma; (b) authentic plasma, *R* enantiomer 37 nmol/l, *S* enantiomer 47 nmol/l.

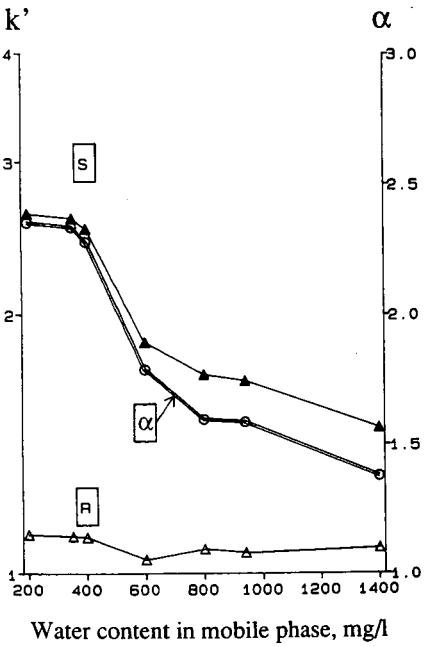


Fig. 3. Effect of water content in the mobile phase on retention (k') and separation factor (α) of (*R*)- and (*S*)-metoprolol. Mobile phase: 10% 1-propanol and 0.1% diethylamine in hexane; temperature, 30°C; flow-rate, 0.75 ml/min.

aration of the four isomers of α-hydroxymetoprolol, the capacity factors of which are plotted in Fig. 4. As can be seen, the water content has to be in the range 600–800 mg/l to enable a complete separation. Even here the *R* forms were influenced to a lesser extent than the *S* isomers. This study was performed with an authentic urine

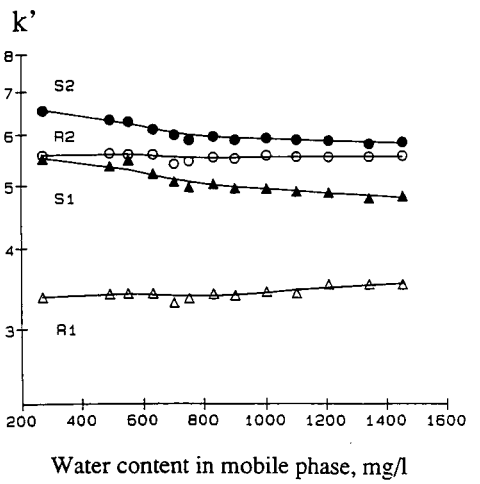


Fig. 4. Effect of water content on retention (k') of the isomers of (*R*)- and (*S*)-α-hydroxymetoprolol. Mobile phase: 10% 1-propanol and 0.1% diethylamine in hexane; temperature, 30°C; flow-rate, 0.75 ml/min.

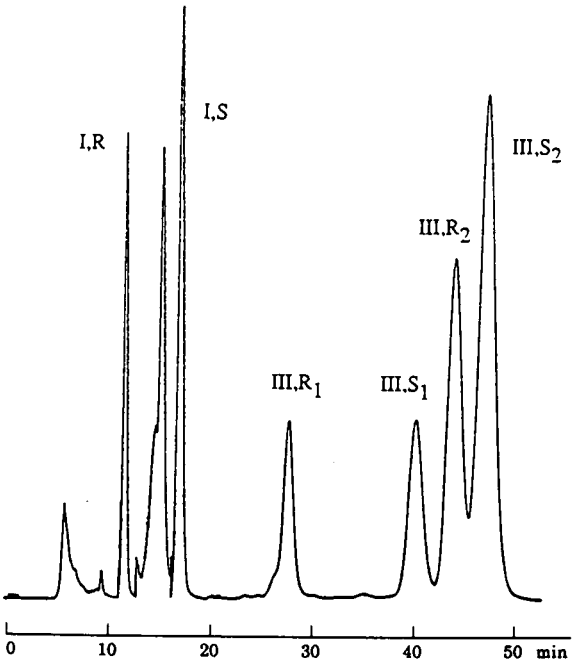


Fig. 5. Chromatogram from a human urine sample containing 1.2 μmol/l metoprolol (I) and 13 μmol/l α-hydroxymetoprolol (III). Integrator attenuation: 512 at start and 128 after 22 min. Mobile phase: 10% 1-propanol, 0.1% diethylamine and 600 mg/l water in hexane; temperature, 25°C; flowrate, 0.5 ml/min.

sample containing the four isomers in different concentrations. *R* and *S* refer to the corresponding metoprolol antipodes.

The concentration of α-hydroxymetoprolol is often low and lower than that of metoprolol in human plasma. The compounds are more easily assayed in human urine. A chromatogram from an authentic sample is shown in Fig. 5, where one pair of metoprolol enantiomers and two pairs of enantiomers of α-hydroxymetoprolol are separated.

In order to track the respective *R* and *S* isomers of α-hydroxymetoprolol, metoprolol racemate and (*S*)-metoprolol were given to two dogs on separate occasions. Chromatograms from the respective administration are shown in Fig. 6a and b and the *R* and *S* peaks can be localized. As can be seen, there is a huge peak in Fig. 6b which appears as a doublet in Fig. 6a. These peaks have the same retention as a diol metabolite, which is postulated as an intermittent degradation product in the dog [11,12] but has not been determined. The chromatogram in Fig. 6a provides evidence of the resolving capability of this Chiracel OD column. The Chiralcel OD columns were found to possess excellent long-term stability and could be used for more than 12 months.

Analytical validation

The absolute recovery was more than 95% for metoprolol and also for the α-hydroxymetabolite after addition of sodium chloride. The metoprolol enantiomers

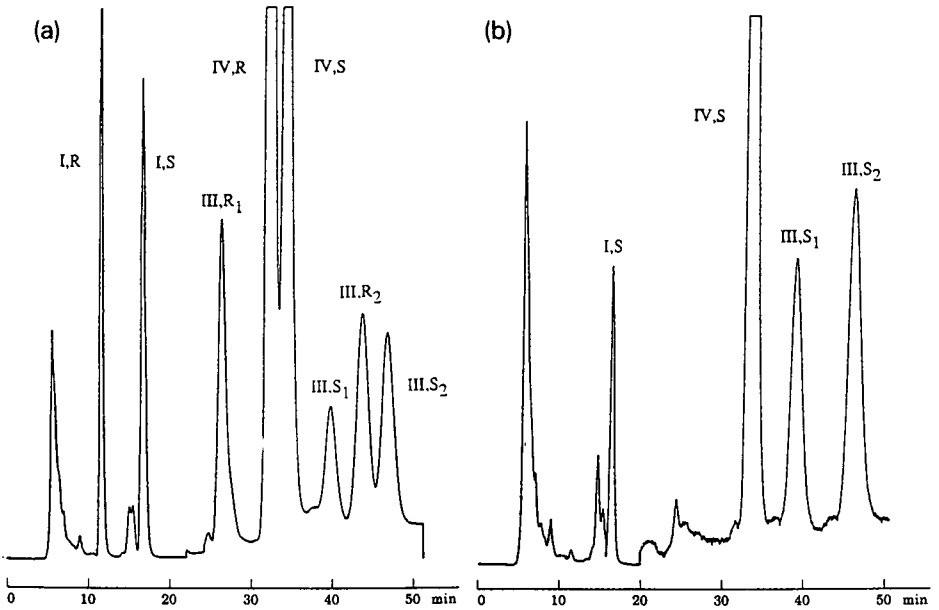


Fig. 6 (a) Chromatogram from dog plasma 0.5 h after dosing with 10 mg/kg of metoprolol racemate. Chromatographic conditions as in Fig. 5. Integrator attenuation: 1024 at start and 64 after 22 min. (b) Chromatogram from dog plasma 0.5 h after dosing with of 5 mg/kg of (*S*)-metoprolol. Chromatographic conditions as in Fig. 5. Integrator attenuation: 512 at start and 128 after 22 min. The numbers I–IV in the chromatograms refer to the compounds listed in Fig. 1.

could be measured in plasma at levels down to 10 nmol/l of each with a relative standard deviation of less than 15%. The calibration graph was linear between 10 and 1000 nmol/l, which covers the therapeutic range. The repeatability (within-day) for the assay of the *R* and *S* enantiomers of metoprolol was 1.3% and 2.2%, respectively, at a level of 100 nmol/l and 3.9% and 6.1%, respectively, at a level of 20 nmol/l (n = 7). The reproducibility (between-day) was 6.4% and 6.1% at 70 nmol/l (n = 10). The α-hydroxymetabolite could be measured at levels down to 50 nmol/l of each isomer. The repeatability for the assay averaged 9.8% at 200 nmol/l (n = 7).

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CHROMSYMP. 2156

Isocratic reversed-phase high-performance liquid chromatographic separation of simple perhalogenated compounds

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ABSTRACT

An isocratic method for the separation of perhalogenated compounds using reversed-phase high-performance liquid chromatography is described. Nine perhalogenated compounds (CCl_4, $CBrCl_3$, $CBrCl_2$, CBr_4, C_2Cl_4, C_2Br_4, C_2Cl_6, $C_2Br_2Cl_4$ and C_2Br_6) were divided into three groups as a function of carbon number and unsaturation and their separations were optimized. Methanol–water mobile phases in different proportions were used and the optimum conditions were determined for each of the groups and for a mixture of all nine compounds, based on resolution and separation factors. These conditions were then used to separate and identify perhalogenated compounds resulting from radiolysis, photolysis or thermolysis of different test solutions. Inversions of retention times were observed for some of the compounds as a function of the mobile phase polarity. This suggests that a gradient elution separation of these compounds would not result in a better separation.

INTRODUCTION

The determination of perhalogenated compounds, often associated with water pollutants, is frequently carried out by gas chromatography [1]. However, many of the possible $C_nBr_yCl_z$ compounds are thermosensitive and decompose or rearrange on injection or during temperature-programed analysis. A high-performance liquid chromatographic (HPLC) procedure would eliminate the thermosensitivity problem and present similar quantitative characteristics.

Little was found in the literature on the use of HPLC for the determination of mixtures of perhalogenated compounds. HPLC determination of C_2Cl_4 [2,3] and C_2Cl_6 [4] using reversed-phase columns with methanol–water eluents has been reported.

This paper describes the separation of several C_1 and C_2 perhalogenated compounds using isocratic elution.

EXPERIMENTAL

Reagents

Most of the perhalogenated compound standards were used as received. Both CBr_4 and C_2Br_6 were sublimed under vacuum in the absence of light immediately

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TABLE I

PERHALOGENATED COMPOUNDS USED AS STANDARDS

Compound	Form	Melting point (°C)[a]	Boiling point (°C)	Source
CCl_4	Liquid	−23	76.5	Merck
$CBrCl_3$	Liquid	5.65	104.7	Eastman-Kodak
CBr_2Cl_2	Semi-solid	22	150.2	Alfa
CBr_4	Solid	90–91	189–190	ICN
C_2Cl_4	Liquid	−19	121	Merck
C_2Br_4	Solid	56.5	226–227	ICN
C_2Cl_6	Solid	186–187 (s)	(186)	Carlo Erba
$C_2Br_2Cl_4$	Solid	220–222	Decomp.	Aldrich
C_2Br_6	Solid	200–210 (d)	Decomp.	K & K Labs.

[a] (s) Sublimes; (d) with decomposition.

before use. The physical properties of the nine perhalogenated standards used are summarized in Table I.

Other perhalogenated compounds were obtained by gamma radiolysis of CBr_4 (3%) in CCl_4 and by photolysis (250–700 nm) of a mixture of C_2Br_6 and Cl_2 in CCl_4.

Chromatographic system

The HPLC system consisted of an Altex Model 110A reciprocating pump, a Rheodyne Model 7010 injection valve with a 10-μl loop and a Schoeffel Spectroflow Model 770 spectrophotometric detector (Kratos Analytical) used with an 8-μl flow cell at 220 nm. The chromatograms were registered on a Model RB-102 recorder (Equipamentos Científicos do Brasil).

Stationary phases

Several columns were tested. Their characteristics are summarized in Table II.

Mobile phases

Methanol (analytical-reagent grade, Merck), acetonitrile (LiChrosolv, Merck) and distilled, deionized water were used in different proportions to prepare mobile phase binary mixtures, which were ultrasonically degassed before use.

TABLE II

CHARACTERISTICS OF THE REVERSED-PHASE COLUMNS USED

Mobile phase, methanol–water (70:30) at 1.0 ml/min; test compound, naphthalene; detection, 220 nm.

Stationary phase	Column dimensions (mm)	Theoretical plates per metre	Asymmetry (10% of peak height)
Vydac TP201, 10 μm (laboratory packed)	124 × 4.6	14 307	1.10
Spherisorb ODS-1, 5 μm (laboratory packed)	100 × 4.6	42 920	1.04
Ultrasphere ODS, 5 μm (Beckman)	250 × 4.6	53 400	0.93

RESULTS AND DISCUSSION

Separations were obtained with all of the columns listed in Table II. Typical results, as a function of mobile phase composition, are shown in Figs. 1 and 2. As can be seen, each group of perhalogenated compounds can be resolved, but with a different mobile phase composition. Of interest is the inversion of elution order observed as the methanol–water composition changes (compare Fig. 1a and d).

Similar results were obtained using acetonitrile–water mixtures. Despite the differences in polarity of methanol and acetonitrile, the separation showed similar retention times and resolutions with similar mobile phase compositions.

Table III summarizes the retention times and resolutions observed with column 3 as a function of mobile phase composition. Mixtures containing more than 50% water were not tested, owing to the very low flow-rates and the resulting long retention times. However, the superposition of the C_1 perhalogenated compounds at a methanol–water composition of 50:50 suggests that inversion of retention times would also occur at higher water contents with this column.

The similar behaviours of all three columns with respect to these polarity changes in interesting, as a recent paper [5] gives a different classification to each of the three stationary phases used. It therefore seems that for such small hydrophobic

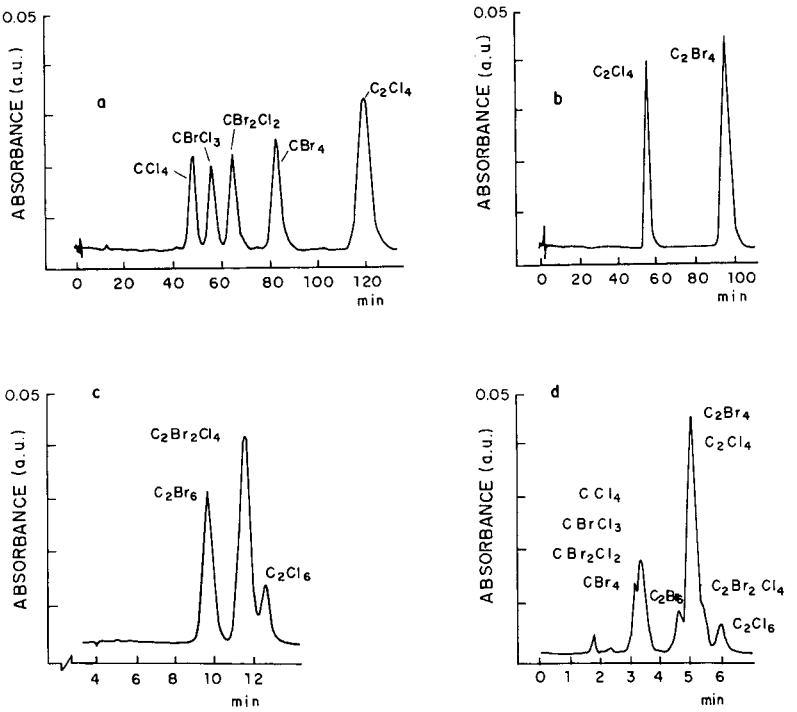

Fig. 1. Chromatograms of various mixtures of perhalogenated compounds obtained with column 1. Stationary phase, Vydac TP210 (124 × 4.6 mm I.D.); injection volume, 10 μl; detection, 220 nm. Methanol–water mobile phase: (a) 22:78 at 1.0 ml/min; (b) 35:65 at 1.0 ml/min; (c) 70:30 at 0.5 ml/min; (d) 70:30 at 0.4 ml/min.

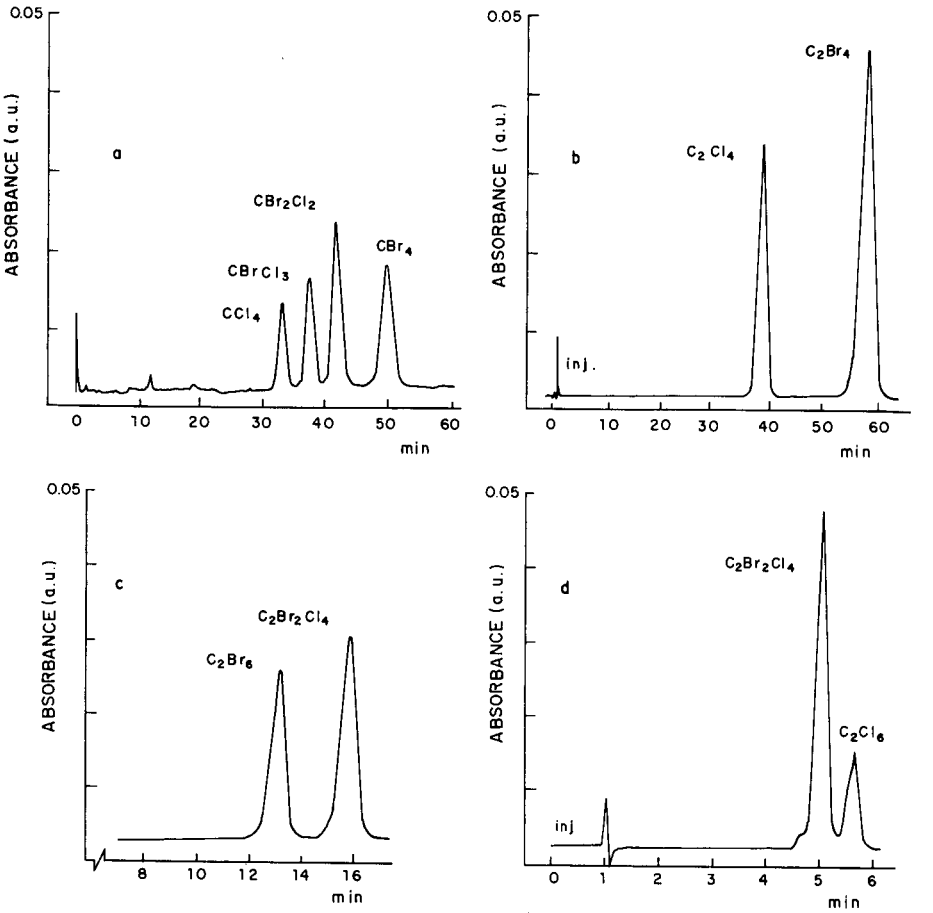

Fig. 2. Chromatograms of various mixtures of perhalogenated compounds obtained with column 2. Stationary phase, Spherisorb ODS-1 (100 × 4.6 mm I.D.); injection volume, 10 μl; detection, 220 nm. Methanol–water mobile phase: (a) 30:70 at 1.0 ml/min; (b) 40:60 at 1.0 ml/min; (c) 60:40 at 1.0 ml/min; (d) 70:30 at 1.0 ml/min.

molecules there is no discernible difference between the monomeric (Ultrasphere), dimeric (Spherisorb) and polymeric (Vydac) phases.

Considering as an objective the separation of as many perhalogenated compounds of the type $C_nBr_yCl_2$ as possible, including those not available as standards, the most appropriate mobile phase composition was fixed as methanol–water (63:37) for use with the Ultrasphere ODS column (Fig. 3). At this composition, the C_1 and C_2 saturated compounds are satisfactorily separated. The high resolution between CBr_2Cl_2 and CBr_4 and between the C_2 perhalogenated standards suggests that other $C_nBr_yCl_2$ compounds could be determined. That this is so is shown in Fig. 4, where two different reaction mixtures reveal the presence of CBr_3Cl, $C_2Br_3Cl_3$ and C_2BrCl_5. These compounds were identified by extrapolation [6] and their identities confirmed by gas chromatography–mass spectrometry.

TABLE III

RETENTION TIMES (t_R, min) AND RESOLUTIONS (R_s) OF PERHALOGENATED COMPOUNDS AS A FUNCTION OF MOBILE PHASE COMPOSITION

Stationary phase, Ultrasphere ODS, 5 μm (250 × 4.6 mm I.D.); mobile phase, methanol–water of various compositions. Flow-rates selected to maintain the pressure at <600 bar.

Compound	Mobile phase composition and flow-rate (ml/min)													
	50:50, 0.7		60:40, 0.8		62.5:37.5, 0.9		63:37, 0.9		65:35, 1.0		70:30, 1.0		80:20, 1.0	
	t_R	R_s	t_R	R_s	t_R	R_s	t_R	R_s	t_R	R_s	t_R	R_s	t_R	R_s
CBr_4	66.3	–	23.1	1.9	21.1	2.0	17.2	2.5	12.3	2.6	9.4	1.9	5.6	2.6
CBr_2Cl_2	69.3	–	24.9	0.9	22.9	1.0	18.8	1.1	13.5	1.3	10.4	1.0	6.1	1.5
$CBrCl_3$	69.3	–	25.8	0.9	23.9	0.9	19.6	1.2	14.1	1.3	10.9	1.2	6.4	1.6
CCl_4	69.3		26.7		24.9		20.5		14.7		11.4		6.8	
C_2Br_4	179.9	5.3	54.1	–	50.3	–	40.1	–	27.3	–	19.2	1.4	9.6	n.c.[a]
C_2Cl_4	150.6		52.2		50.3		40.1		27.3		20.4		10.7	
C_2Br_6	247.0	4.8	60.4	6.2	54.1	6.3	41.8	6.8	26.8	7.0	18.3	5.2	8.3	n.c.
$C_2Br_2Cl_4$	298.0	2.3	75.2	3.6	68.9	3.5	53.4	3.6	34.1	4.1	23.3	4.1	10.3	n.c.
C_2Cl_6	329.5		85.3		80.0		62.0		39.2		27.1		11.9	

[a] n.c. = Not calculated.

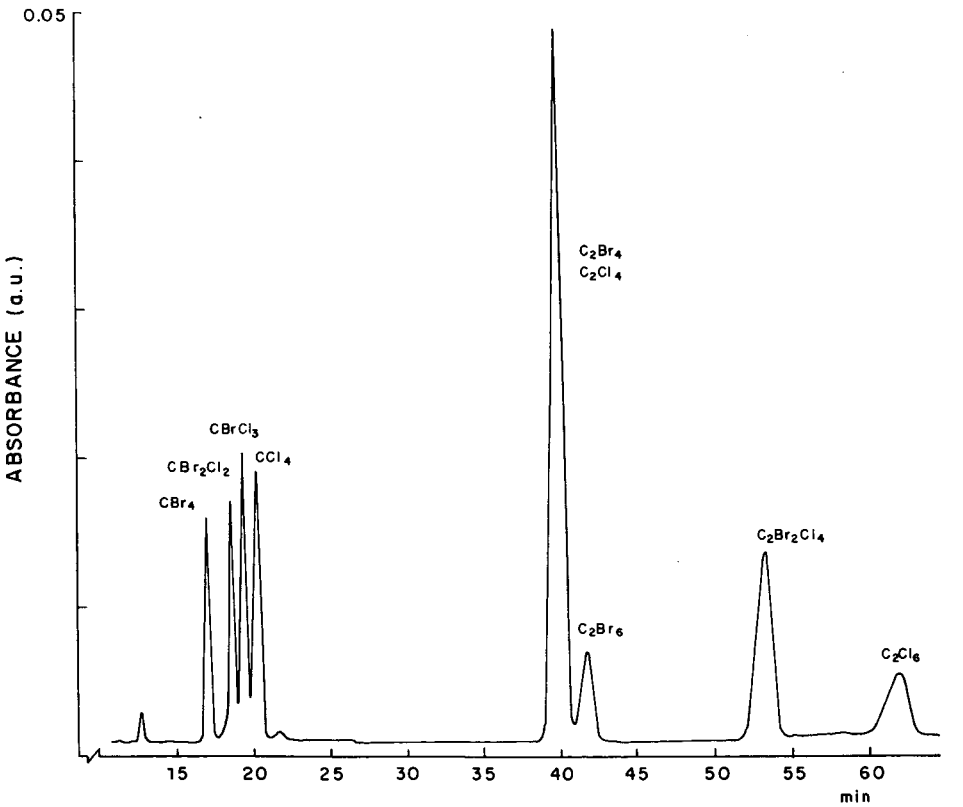

Fig. 3. Chromatograms of a mixture of perhalogenated compounds obtained with column 3. Stationary phase, Ultrasphere ODS (230 × 4.6 mm I.D.); mobile phase, methanol–water (63:37) at 0.9 ml/min; injection volume, 10 μl; detection, 220 nm.

If C_2 unsaturated compounds are present in the reaction mixtures a second chromatogram is run, at a lower water content, to permit the identification of these compounds, as with the chosen isocratic phase [methanol–water (63:37)] all C_2 unsaturated compounds are predicted to be superimposed.

The determinations reported here were applied to a large number of reaction mixtures of the types exemplified by Fig. 4. The retention times are remarkably reproducible, even after a chlorodimethyloctadecylsilane treatment of column 3.

With the methanol–water (63:37) system, the mass distribution ratios, k, for the C_1 compounds lie between 6 and 8 and those for the C_2 saturated perhalogenated compounds between 16 and 26. The latter higher values could be reduced by altering

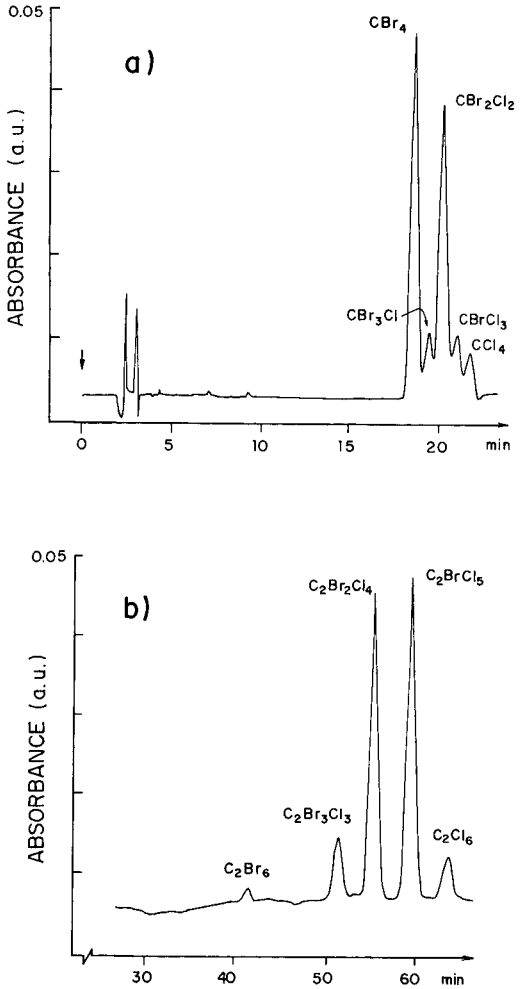

Fig. 4. Chromatograms of several reaction mixtures obtained with column 3. Stationary phase, Ultrasphere ODS (250 × 4.6 mm I.D.); mobile phase, methanol–water (63:37) at 0.9 ml/min; injection volume, 10 μl; detection, 220 nm. (a) Identification of gamma radiolysis (50 kGy) products from a solution of 3% CBr_4 in CCl_4; (b) identification of the UV photolysis products of a solution of C_2Br_6 in Cl_2-saturated CCl_4.

the mobile phase polarity, but this would sacrifice the separation of these compounds, and also the resolution of the C_1 compounds, if isocratic elution is used. On the other hand, the sensitivity of the retention times to the mobile phase composition, with possible superposition or even inversion of the elution order, suggests that gradient elution is not the solution to the problem presented by the long retention times of the C_2 compounds.

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Retention of some ethylenediamine oligomers in reversed-phase chromatography

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ABSTRACT

The retention behaviour of four ethylenediamine oligomers containing two, three, four and five monomer units was studied in reversed-phase thin-layer chromatography using various alcohols, salts, acetic acid and ammonia as eluent additives. It was established that with salt-free eluents the oligomers showed very high retention. The retention decreased with increasing salt concentration in the eluent. This phenomenon may be due to the dissociation-suppressing effect of the salts and/or to the competition for the free silanol groups on the silica surface uncovered by the impregnating agent. The salts with higher activity coefficients had a greater effect on the retention whereas lower dielectric constants of the alcohols decreased their influence on the retention. The results suggest that to obtain the minimum retention of ethylenediamine oligomers (and probably other polyamines), the use of a salt with a high activity coefficient and organic mobile phases with high dielectric constants can be recommended.

INTRODUCTION

The chromatographic separation of amines is complicated by the strong silanophilic interaction between the amino groups and the silanol groups of the stationary phase. Nevertheless, several chromatographic techniques have been developed for the analysis of amines. Adsorption and reversed-phase thin-layer chromatography (RP-TLC) [1] and soap chromatography [2,3] have also been applied to separate amine mixtures. Cation [4,5] and anion [6] exchangers could also produce good separations. In adsorption chromatography, the silanophilic interactions have been modified by treating the stationary phase, *e.g.*, with phenols [7] or transition metal complexes [8].

Various eluent additives have a considerable effect on the retention of polar compounds in (RP-TLC). When the solute contains one or more dissociable polar substituents, the pH of the eluent strongly modifies the retention [9,10]. Salts may exert a similar effect on the retention. A salt adsorbed on the silica surface changes the retention characteristics of the sorbent [11], and salts added to the eluent markedly

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change the reversed-phase mobility of polar solutes [12]. The retention of some methylated amino acids [13] and crown ether derivatives [14] decreased non-linearly with increasing salt concentration in the eluent. The type of cations also influenced the retention of some aniline derivatives in RPTLC [15]. With the antimicrobial agent chlorhexidine {1,1-hexamethylene-bis[5-(4-chlorophenyl)biguanidine]} it was established that the ionic strength has a greater effect than the pH value on the retention [16]. As the apparent dissociation constant of salts depends strongly on the dielectric constant of the solvent [17] and most reversed-phase eluents contain an organic mobile phase, which decreases the dielectric constant, it is reasonable to suppose that the effect of salts also depends on the composition of the eluent.

The objectives of this work were to study the RP-TLC behaviour of some ethylenediamine oligomers and to elucidate the relationship between the role of salts and organic mobile phases on the retention. Ethylenediamine oligomers are used as epoxy resin hardeners, but only a few methods have been developed for their separation [18–21].

EXPERIMENTAL

Diethylenetriamine (compound I), triethylenetetramine (II), tetraethylenepentamine (III) and pentaethylenehexamine (IV) were synthesized at the EGIS Pharmaceutical Works (Budapest, Hungary). The compounds were chromatographically pure. All other reagents were of analytical-reagent grade. Silufol plates (Kavalier, Sklárny, Czechoslovakia) were impregnated by overnight predevelopment in the eluent paraffin oil-*n*-hexane (5:95, v/v). Silufol plates are prepared from large-pore silica with a starch binder having a fairly low developing time. The compounds were separately dissolved in methanol at a concentration of 5 mg/ml and 2-μl volumes of these solutions were spotted on the plates. Water–methanol (11:9, 9:11 and 7:13, v/v), water–ethanol (12:8, 11:9 and 10:10, v/v) and water–1-propanol (14:6, 13:7 and 12:8, v/v) were used as eluents. The salt concentration in the eluent was 0–4.5 *M* in steps of 0.5 *M* for lithium chloride and ammonium acetate and 0–2.25 *M* in steps of 0.25 *M* for lithium acetate and ammonium chloride. To study the effect of pH on retention, acetic acid and ammonia were added to the eluents in the concentration range 0–4.37 *M* in steps of 0.88 *M*. After development the plates were dried at 105°C and the ethylenediamine oligomers were detected with ninhydrin. To increase the sensitivity of detection, the plates developed in alkaline eluents were first sprayed with 2 *M* acetic acid. Each experiment was run in quadruplicate.

The R_M values of the ethylenediamine oligomers were calculated according to the equation [9]

$$R_M = \log(1/R_F - 1) \quad (1)$$

To elucidate the individual effects of eluent additives on the R_M values of ethylenediamine oligomers, stepwise regression analysis [22] was applied to select the independent variables that influence the R_M value significantly. The R_M value in eqn. 1 was taken as dependent variable. The total salt (independently of the type of salt), acetic acid and ammonia concentrations (three variables) and as combined variables (each salt concentration multiplied by each alcohol concentration = 12 variables)

served as independent variables. The inclusion of the combined variables in the calculation was motivated by the theoretical considerations outlined in the Introduction and the facts that the ions account mainly for the modification of the retention of polar compounds and that the dissociation of salts is influenced by the dielectric constant of the eluent, which depends on the type and concentration of the alcohols. The stepwise regression analysis was carried out separately for each ethylenediamine oligomer, the significance level of the acceptance of the individual independent variables was set at 95% and the number of accepted variables was not limited.

To find the relationship between the effect of significant variables on the retention and physico-chemical parameters, correlations were calculated between the regression coefficient values of the independent variables selected by the stepwise regression analysis and the activity coefficient of salt, the number of ethylenediamine units and the dielectric constant of the alcohols. The dielectric constants and the activity coefficients of lithium salts and ammonium chloride were taken from refs. 23, 24 and 25, respectively. The regression coefficients in Tables I–IV served as dependent variables. The independent variables were the number of ethylenediamine units in the oligomer (x_1), the activity coefficient of the corresponding salts (x_2), the dielectric constant of the alcohols (x_3) and two combined variables, $x_4 = x_2/x_4$ and $x_5 = x_1x_4$. The inclusion of the last two variables was motivated by the assumptions outlined above.

The calculation was carried out with the same stepwise regression analysis programme under the same conditions. Data for which the relative standard deviation between parallel determinations was higher than 6% were omitted from the calculations.

RESULTS AND DISCUSSION

The ethylenediamine oligomers remained at the start or very near the start with each salt-free eluent, even with the most acidic and alkaline eluents. This indicates that these highly polar compounds bind very strongly to the free silanol groups of the silica surface in both dissociated and undissociated forms. The retention of the ethylenediamine oligomers decreased linearly with increasing salt concentration in the eluent (Fig. 1) and the effect was higher for compound IV than for compound I. This observation can be explained by the assumption that the longer oligomers occupy more adsorption sites than the smaller oligomers on the silica surface and therefore their retention changes more rapidly when the ions saturate the adsorption sites. The phenomenon seems to be of competitive character. The retention order does not follow the general rule of reversed-phase chromatographic separations in neutral and alkaline eluent systems: the most polar compound IV is the most strongly retained followed by compounds III, II and I. This anomalous retention behaviour can also be explained by the suppositions outlined above.

The concentration of the organic mobile phase has a relatively small effect on the retention (Fig. 2). This observation again proves the strong adsorption capacity of ethylenediamine oligomers. Acidification of the eluent also decreased the retention of each oligomer (Fig. 3). At higher acetic acid concentrations the retention order changes and at the highest salt and acid concentrations it is totally reversed (that is, it follows the general rule): the longest oligomer shows the lowest retention and the

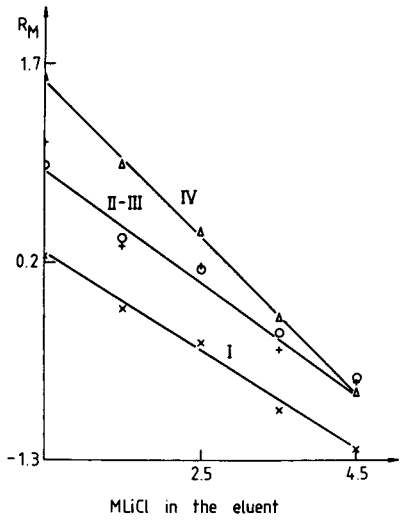

Fig. 1. Effect of LiCl concentration on the R_M value of ethylenediamine oligomers (40 vol.% ethanol). I = diethylenetriamine; II = triethylenetetramine; III = tetraethylenepentamine; IV = pentaethylenehexamine.

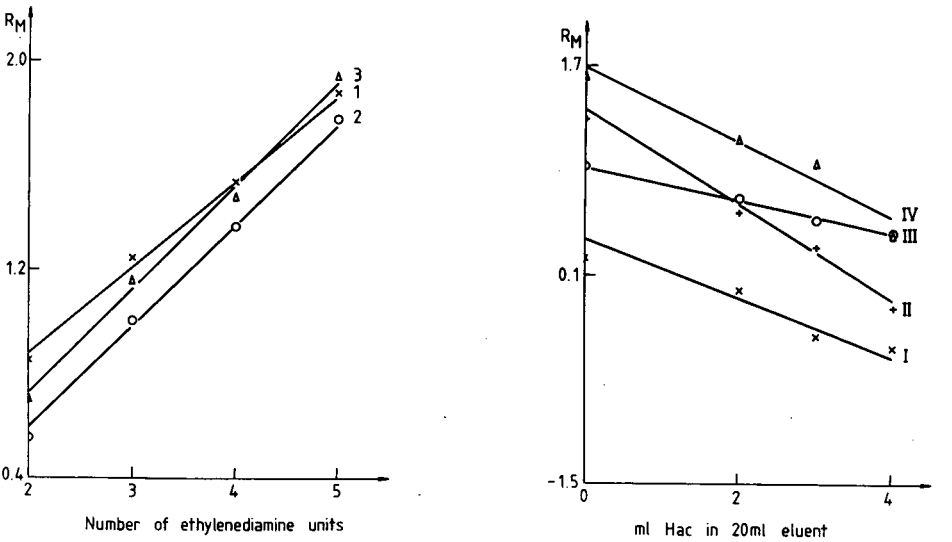

Fig. 2. Effect of the number of ethylenediamine monomer units on the R_M value at a 0.375 *M* lithium acetate concentration. Methanol concentration: 1, 45 vol.%; 2, 55 vol.%; 3, 65 vol.%.

Fig. 3. Effect of acetic acid (Hac) concentration on the R_M value of ethylenediamine oligomers at 40 vol.% ethanol and 0.5 *M* LiCl concentration. I = diethylenetriamine; II = triethylentetramine; III = tetraethylenepentamine; IV = pentaethylenehexamine.

TABLE I

EFFECT OF ELUENT COMPOSITION ON THE R_M VALUE OF DIETHYLENETRIAMINE: RESULTS OF STEPWISE REGRESSION ANALYSIS

$R_M = a + b_1C_1C_2 + b_2C_1C_3 + b_3C_1C_4 + b_4C_5C_2 + b_5C_5C_3 + b_6C_5C_4$

where C_1 = vol.% methanol in the eluent, C_2 = NH_4Cl concentration in the eluent (M), C_3 = LiCl concentration in the eluent (M), C_4 = CH_3COOLi concentration in the eluent (M) and C_5 = vol.% ethanol in the eluent.

$n = 31$; $a = -8.40$; $F = 23.52$; $r^2 = 0.8547$.

Parameter	Number of independent variable					
	1	2	3	4	5	6
b	−0.87	−0.29	0.94	−1.36	−0.53	1.07
s_b	0.18	0.08	0.23	0.22	0.09	0.36
Path coefficient (%)	17.01	12.12	14.40	21.15	20.77	10.27

smallest oligomer is the most strongly retained. This result is probably due to two effects, *viz.*, the compounds are in a highly dissociated state and the adsorption centres on the silica surface are totally occupied by the ions.

The results of stepwise regression analysis are compiled in Tables I–IV. The equations selected by the stepwise regression analysis fit the experimental data well, the significance level is in each instance being over 99.9% (see F values). The equations account for about 85–95% of the total variance (see r^2 values), that is, the change in the independent variables (chromatographic conditions) explains most of the change in the dependent variable (retention of the ethylenediamine oligomers). The results support our previous suppositions; the combined variables explain most of the total variance, that is, the effects of salt and organic modifier are not independent of each other. This result lend support to the assumption that only the dis-

TABLE II

EFFECT OF ELUENT COMPOSITION ON THE R_M VALUE OF TRIETHYLENETETRAMINE: RESULTS OF STEPWISE REGRESSION ANALYSIS

$R_M = a + b_1C_1C_2 + b_2C_1C_3 + b_3C_1C_4 + b_4C_5C_2 + b_5C_5C_3 + b_7C_6C_3 + b_8C_7 + b_9C_8$

where C_6 = vol. % 1-propanol in the eluent, C_7 = acetic acid concentration in the eluent (M), C_8 = salt concentration in the eluent (M) and other symbols are as in Table I.

$n = 32$; $a = 80.68$; $F = 37.08$; $r^2 = 0.9280$.

Parameter	Number of independent variable							
	1	2	3	4	5	7	8	9
b	−1.30	−0.34	0.71	−1.65	−0.63	−0.58	−29.6	−9.67
s_b	0.16	0.08	0.21	0.20	0.09	0.20	4.29	3.79
Path coefficient (%)	17.64	9.70	7.61	17.96	17.73	6.51	15.62	7.22

TABLE III

EFFECT OF ELUENT COMPOSITION ON THE R_M VALUE OF TETRAETHYLENEPENTAMINE: RESULTS OF STEPWISE REGRESSION ANALYSIS

$R_M = a + b_1C_1C_2 + b_2C_1C_3 + b_4C_5C_2 + b_5C_5C_3 + b_7C_6C_3 + b_8C_7 + b_{10}C_6C_2$

Symbols as in Tables I and II.

$n = 32$; $a = 86.00$; $F = 18.33$; $r^2 = 0.8424$.

Parameter	Number of independent variable						
	1	2	4	5	7	8	10
b	−1.25	−0.57	−1.53	−0.88	−0.82	−31.24	−2.36
s_b	0.30	0.12	0.30	0.12	0.29	6.31	0.61
Path coefficient (%)	12.49	13.94	15.36	22.80	8.49	15.21	11.71

sociated ions of the salts influence the retention, and the extent of dissociation depends on the composition of the eluent.

Ammonium acetate, having the lowest activity coefficient [25], has no significant effect on the retention. The acetic acid concentation significantly decreases the retention of the longer oligomers but not of the smallest oligomer. This result can be explained by the supposition that the increasing number of polar groups makes the compound more sensitive to the acidity of the environment.

The effects of the various combined variables on the retention of ethylenediamine oligomers are commensurable (see path coefficients), but the absolute value of the regression coefficient differs considerably. The path coefficients (normalized slope values) are dimensionless numbers reflecting the relative impact of the independent variables on the dependent variable. As they are normalized values they are independent of the orginal dimension of the independent variables.

The activity coefficients of the salts and the dielectric constants of the alcohols explain well their effect on the retention of the ethylenediamine oligomers (Table V).

TABLE IV

EFFECT OF ELUENT COMPOSITION ON THE R_M VALUE OF PENTAETHYLENEHEXAMINE: RESULTS OF STEPWISE REGRESSION ANALYSIS

$R_M = a + b_1C_1C_2 + b_2C_1C_3 + b_3C_1C_4 + b_4C_5C_2 + b_5C_5C_3 + b_7C_6C_3 + b_8C_7 + b_{10}C_6C_3 + b_{11}C_9$

where C_9 = ammonia concentration in the eluent (M) and other symbols are as in Tables I and II.

$n = 31$; $a = 112.91$; $F = 48.72$; $r^2 = 0.9543$.

Parameter	Number of independent variable								
	1	2	3	4	5	7	8	10	11
b	−1.47	−0.64	1.80	−1.75	−0.96	−0.84	−31.07	−2.60	−34.33
s_b	0.22	0.08	0.31	0.21	0.09	0.20	4.42	0.49	6.38
Path coefficient (%)	10.62	12.95	9.41	13.30	18.54	6.58	11.47	8.53	8.59

TABLE V

EFFECT OF ACTIVITY COEFFICIENTS OF SALTS (*AC*) AND DIELECTRIC CONSTANTS OF THE ALCOHOLS (*DC*) ON THEIR IMPACT ON THE RETENTION (*b*) OF ETHYLENEDIAMINE OLIGOMERS: RESULTS OF STEPWISE REGRESSION ANALYSIS

$b = a + b_1AC + b_2AC/DC$

$n = 25$; $a = 14.92$; $F = 48.69$; $r^2 = 0.8157$.

Parameter	Number of independent variable	
	1	2
b	−18.68	−60.24
s_b	2.60	16.76
Path coefficient (%)	66.66	33.34

The equation fits the experimental data well, the significance level being over 99.9% (see F value). The changes in the independent variables account for about 82% of the retention changes of the ethylenediamine oligomers (see r^2 value). The data prove that the salts with higher activity coefficients decrease the retention more strongly (higher amount of dissociated ions) and the alcohols with lower dielectric constants decrease the effect of salts more strongly.

Our data are in good agreement with the solvophobic theory in ref. 26, according to which three types of interactions may occur between the solutes and the stationary phases, namely coulombic, hydrogen bonding and hydrophobic (solvophobic). As the ethylenediamine oligomers contain both polar (amino groups) and apolar (ethylene groups) substructures and the results of stepwise regression analysis indicate significant effect of salts, organic modifiers and pH on the retention, we assume the each type of interaction may have some influence on the retention behaviour of the ethylenediamine oligomers.

In conclusion, the calculations suggest that to decrease the retention of polar compounds in reversed-phase chromatography, salts with high activity coefficients and organic modifiers with high dielectric constants are strongly recommended.

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CHROMSYMP. 2171

Determination of polymeric hindered amine light stabilizers in polyolefins by high-performance liquid chromatography

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ABSTRACT

Thin-layer and high-performance liquid chromatography procedures were used for the identification and quantitative determination of the polymeric hindered amine light stabilizers Chimassorb 944 and Cyasorb UV 3346 in polyolefins. After extraction from the polymer by the appropriate solvents, the hindered amine light stabilizers solution was qualitatively tested by thin-layer chromatography and successively analysed with a separation technique described as size-exclusion non-aqueous reversed-phase chromatography using traditional high-performance liquid chromatography equipment. The stationary phase was a non-polar styrene–divinylbenzene copolymer. The mobile phase was tetrahydrofuran containing diethanolamine. Both hindered amine light stabilizers were eluted at times ranging from 10 to 19 min and detected by UV at 239 nm, also showing a specific molecular mass distribution of their telomers. Interference due to other stabilizers was determined by a second elution of the same extraction solution after selective treatment on silica-gel cartridge.

INTRODUCTION

Since the commercial introduction of hindered amine light stabilizers (HALSs), increasing interest has been shown in studies of their protective mechanisms and of their practical use in industrial stabilizing packages for polyolefins, even at low concentrations and in the presence of other types of additives. Several analytical investigations aiming at their identification and quantitative determination in polymers have been carried out. Among the various analytical techniques, the liquid chromatographic approach appears to be one of the most adequate for both specificity and sensitivity.

In the case of monomeric HALSs, several methods have appeared in the literature concerning common commercial products [1–4]. In the field of polymeric HALSs, some spectroscopic [5,6], pyrolysis–gas chromatographic (GC) [7] and, more recently, high-performance liquid chromatographic (HPLC) [8–10] procedures have been proposed for the commercial stabilizers Chimassorb 944 and Tinuvin 622 (Ciba-Geigy, Basle, Switzerland).

This paper describes liquid chromatographic procedures suitable for identification and quantitative determination of Chimassorb 944 and Cyasorb UV 3346 (Cyanamid) polymeric HALSs in crystalline polyolefins. The proposed procedures provide considerable advantages in comparison with other available methods, such as:

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the requirement for traditional thin-layer chromatographic (TLC) and HPLC equipment (isocratic conditions and UV detector); the low detection limit (down to 1.0 μg of Chimassorb 944 and 0.5 μg of Cyasorb UV 3346, corresponding to a concentration of 10 and 5 ppm in the polymer sample, respectively) as well as good recovery (about 95%) from the polymer; the removal of interference from other additives used along with polymeric HALSs commercial stabilization packages; and qualitative information on the molecular mass distribution profile of the HALSs as produced, and after polymer extraction.

EXPERIMENTAL

HPLC apparatus

A Varian Model 5500 liquid chromatograph was employed, equipped with a Rheodyne 7125 injection valve and a selection of 100-, 200- or 500-μl loops, a diode array Varian Polychrome 9060 detector, and a DS 651 data station. The analytical columns were Waters Ultrastyragel 1000 Å (30 × 0.78 cm I.D.; code 8552) and Waters Ultrastyragel 500 Å (30 × 0.78 cm I.D.; code 8551) connected in series. Moreover, the injection system may include a direct injection pre-selective silica-gel cartridge (Alltech code 20974) and a Luer-Hub syringe (5 ml).

TLC apparatus

Pre-coated TLC plates (20 × 20 cm) with a layer thickness of 0.25 mm of silica gel 60 F254 (code 11798, Merck, Darmstadt, Germany) were used. A Camag Linomat IV sample application device was employed for sample deposit.

Reagents

Chimassorb 944 and Tinuvin 770 are commercial products supplied by Ciba-Geigy; Chimassorb 905 is a monomeric HALS not commercially available and was provided by Ciba-Geigy; Cyasorb UV 3346 is a commercial product supplied by Cyanamid (Wayne, NJ, U.S.A.). *n*-Hexane, ethyl acetate, chloroform, 2-propanol, methanol, ammonium hydroxide, xylene, tetrahydrofuran (THF) (stabilized) and diethanolamine were all reagent grade obtained from C. Erba (Milan, Italy). Tetrahydrofuran (unstabilized), HPLC grade, was supplied by Merck.

Extraction procedure

A weighed polymer sample (2.5 g) is dissolved in 80 ml of xylene by refluxing for 30 min under stirring. After cooling to about 100°C, 160 ml of 2-propanol are added through the condenser under vigorous stirring. The precipitated polymer is filtered at room temperature under vacuum, and washed with xylene–2-propanol (1:2, v/v). The filtered solution is evaporated to dryness in Rotavapor and the residue dissolved and brought to 10 ml with tetrahydrofuran (stabilized). If the polymer sample is in the form of fibers, powders or flakes, a simplified extraction procedure might be applied, as follows: 10 g of polymer are extracted with 50 ml of tetrahydrofuran at room temperature under stirring for about 1 h; this solution may be directly injected into the chromatograph.

TLC qualitative analysis

A portion (5–50 μl, according to the expected sample concentration) of extraction solution and equivalent amounts of standard solutions of pure additives are spotted on a pre-coated TLC plate. The plate is eluted in the ascending mode with *n*-hexane–ethyl acetate (7:3, v/v), and after drying at room temperature eluted again with chloroform–methanol (92.5:7.5, v/v), which has been previously saturated by shaking it with a 28° Bè ammonium hydroxide solution.

The first elution run (16–17 cm) removes interferences from extracted oligomers and other stabilizers such as phenols, phosphites, phosphonites, thioesters etc. The second elution run (10–11 cm) allows Chimassorb 944 and Cyasorb UV 3346 to separate.

After drying, the chromatograms are developed by chlorination with chlorine gas or *tert.*-butyl hypochlorite, and then sprayed with a potassium iodide (0.5%, w/v) and soluble starch (0.5%, w/v) mixed acqueous solution [11,12]. Dark violet–blue spots appear on a pale blue background. Chimassorb 944 and Cyasorb UV 3346 are identified as sequences of spots at R_F = 0.3–0.75 and R_F = 0.12–0.5, respectively.

HPLC analysis

The sample solution and proper external standard solutions in THF were injected into the chromatograph by means of a sample loop. The chromatographic conditions were as follows. Mobile phase: 0.02 *M* diethanolamine in HPLC-grade THF. Flow-rate: 1.0 ml/min. Column temperature: 40°C. Detector wavelength: 239 nm. Data station: area integration in the "group peaks" mode from 10 to 16.5 min elution time and "horizontal forward baseline" from 10 to 22 min. Typical chromatograms of two Chimassorb 944 are shown in Fig. 1. Fig. 2 shows the chromatogram of the extracted solution of an actual stabilized polymer including Chimassorb 944 and some additional antioxidants.

Retention times of Chimassorb 944 range from 10 to 19 min. On the other hand, the most frequently used antioxidants in commercial polymers have retention times of 14–20 min for instance Irganox 1010 is eluted at about 14 min retention time.

RESULTS AND DISCUSSION

As clearly shown in Fig. 2A, interference may arise from several common polymer additives. When this is the case, accurate quantitative evaluation of Chimassorb 944 requires removal of these interfering peaks. To this purpose, a second injection of the extracted solution is performed under exactly the same chromatographic conditions, but after selective treatment of the solution on a silica cartridge (see Experimental). One cartridge is used for each sample injection. Such treatment causes Chimassorb 944 to be completely retained on silica but allows elution of all the other stabilizers, thus quantitatively evaluated as the overall interfering peak area.

Fig. 2B shows the same sample as Fig. 2A after silica cartridge pre-treatment.

The concentration of Chimassorb 944 is calculated by subtracting the total interfering area originating from the second sample injection from the total area of the first, and by then comparing the resulting difference with the standard area, following the usual external standard method.

The same experimental conditions can be applied to the qualitative and

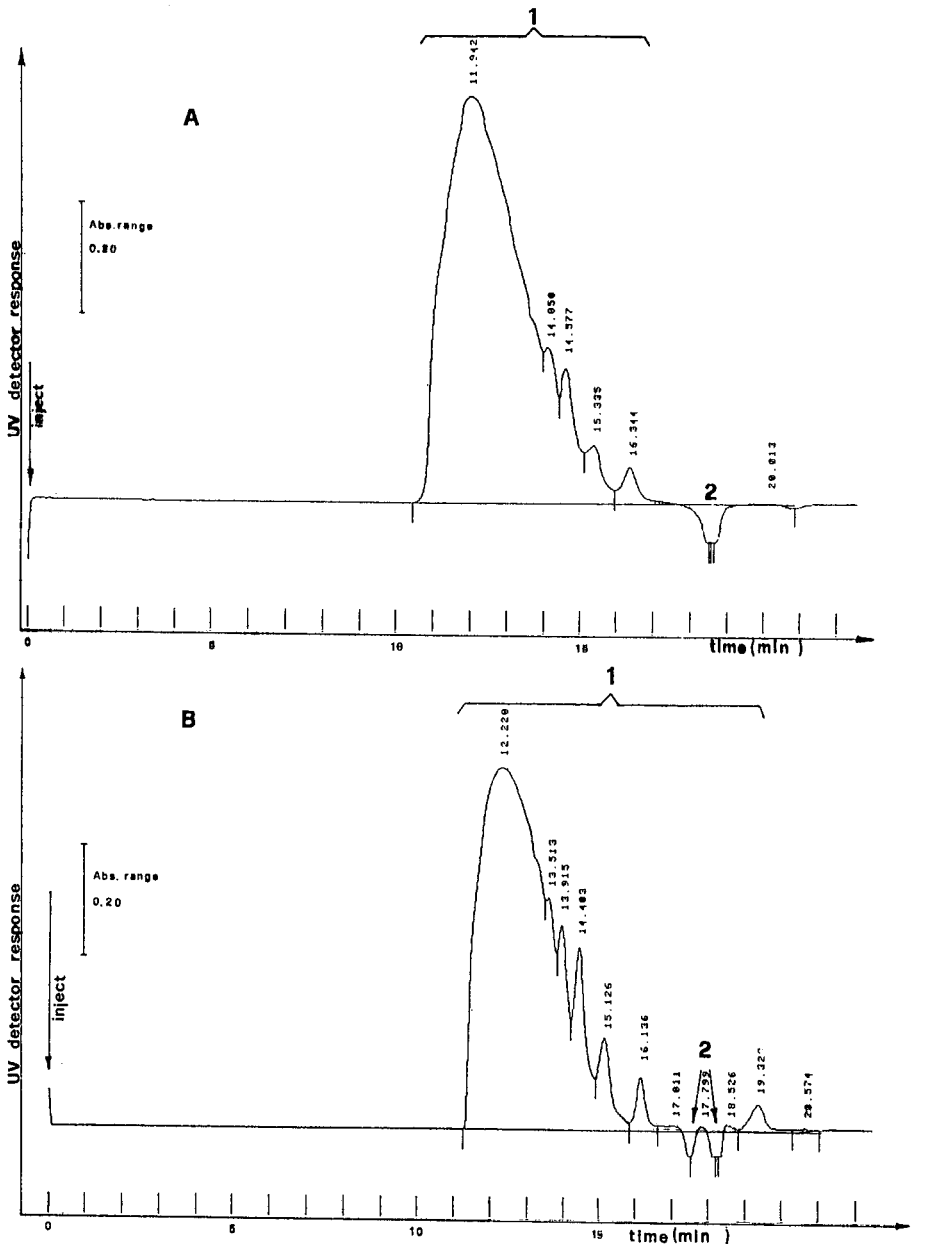

Fig. 1. (A) Chromatogram of Chimassorb 944 (commercial product A), 125 μg. (B) Chromatogram of Chimassorb 944 (commercial product B), 125 μg. Peaks: 1 = Telomers of Chimassorb 944; 2 = THF solvent (negative peak).

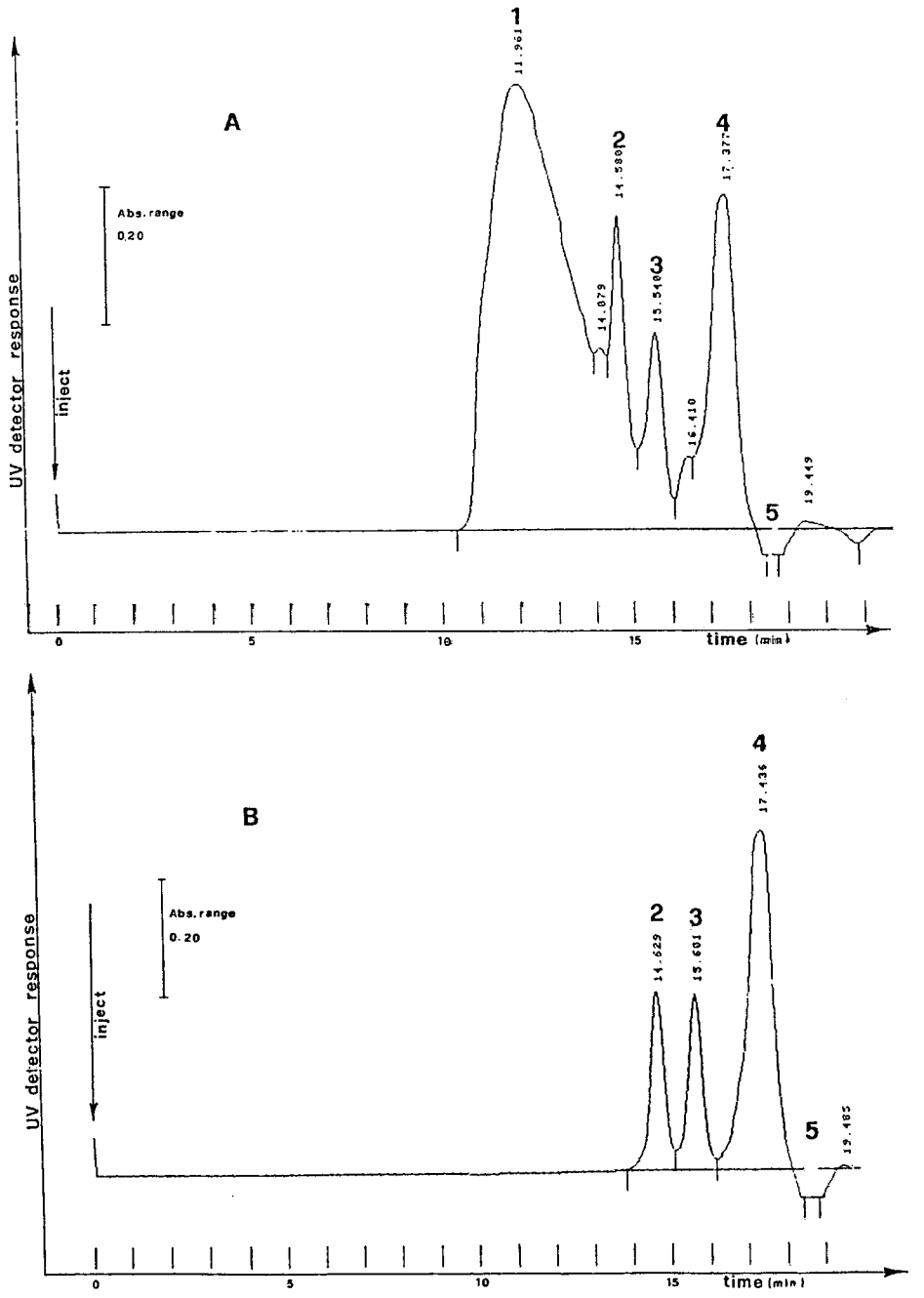

Fig. 2. (A) HPLC trace of a polymer sample extract (direct injection). (B) HPLC trace of a polymer sample extract (injection after pre-treatment on silica gel cartridge). Peaks: 1 = Chimassorb 944 commercial product A (125 μg); 2 = Irganox 1010 (75 μg); 3 = Irganox 1076 (135 μg); 4 = BHT (tetrahydrofuran stabilizer); 5 = THF solvent (negative peak).

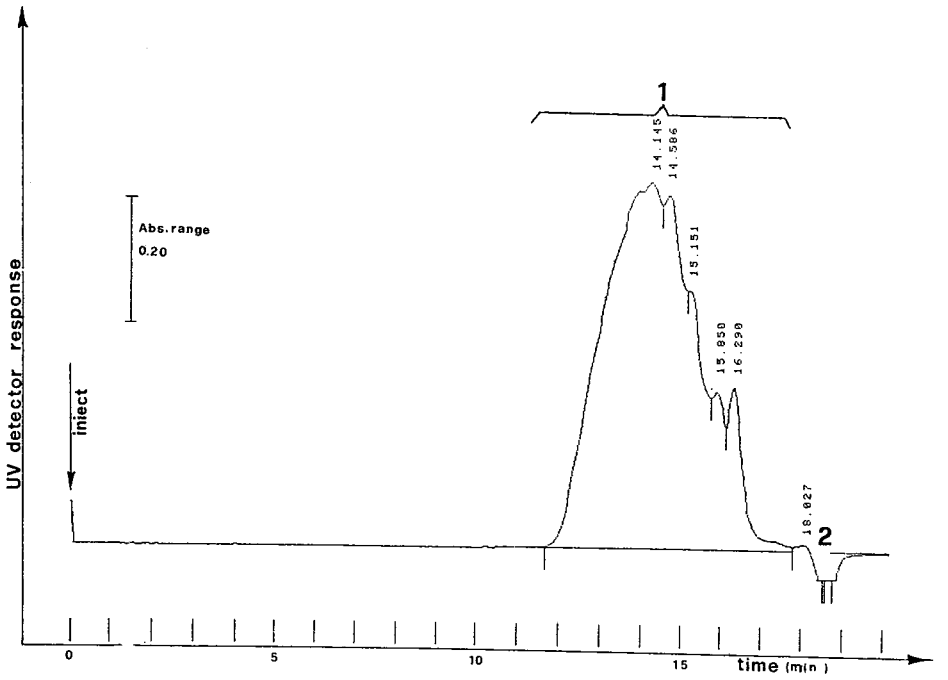

Fig. 3. Chromatogram of Cyasorb UV 3346. Peaks: 1 = telomers of Cyasorb UV 3346 (62 μg); 2 = THF solvent (negative peak).

quantitative evaluation of Cyasorb UV 3346 polymeric HALS of similar structure (Fig. 3).

The chromatograms in Figs. 1 and 3 provide useful qualitative information on the molecular mass distribution profile of the above HALS, with a better selectivity towards the medium–low molecular masses.

Fig. 4 shows the chromatogram of Chimassorb 944 and a mixture of Tinuvin 770 and Chimassorb 905 monomeric HALSs as reference compounds with known molecular masses of 427 and 2174, respectively.

Finally, a comparison of Figs. 1 and 2 demonstrates that Chimassorb 944 extracted from the polymer in the present conditions (Fig. 2) shows a similar, unchanged molecular mass distribution profile when compared to the original additive (Fig. 1).

The extraction procedure and the chromatographic conditions described in the present study seem to be adequate, with relevant adjustments, for the analysis of other polymeric HALS —even those which are not detected by UV (*e.g.*, Tinuvin 622, Ciba-Geigy), provided that, in this instance, the UV detector is replaced by a refractive index detector.

Additional work is in progress in our laboratory now, aimed at extending the present analytical conditions, and at developing proper methods to determine Tinuvin 622 and other interesting polymeric HALSs in polyolefins.

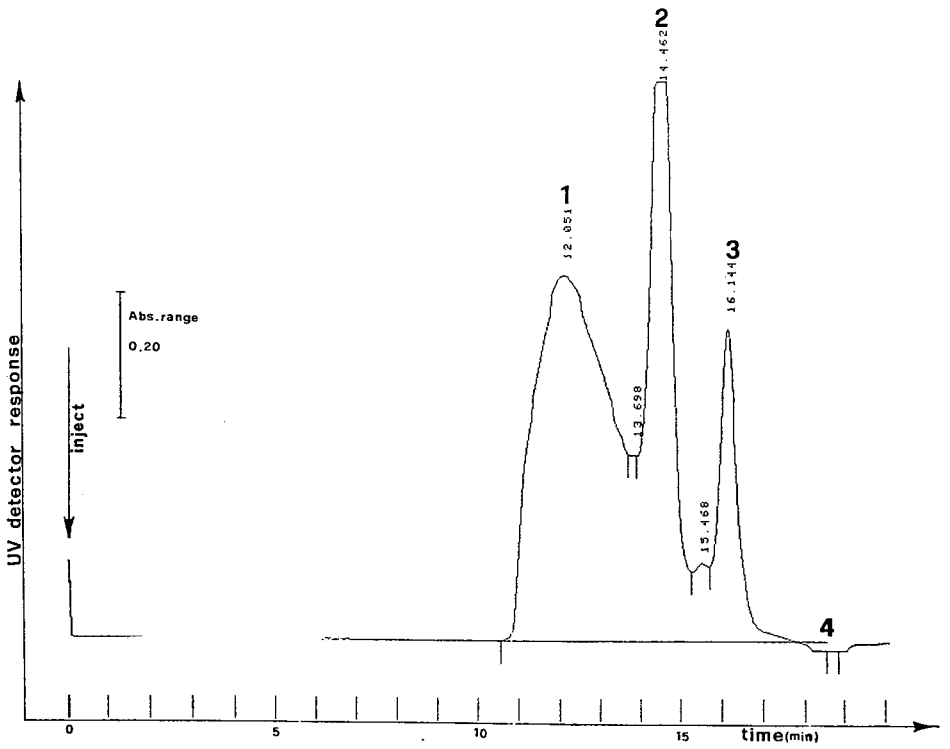

Fig. 4. Chromatogram of a mixture of Chimassorb 944, Chimassorb 905 and Tinuvin 770. Peaks: 1 = Chimassorb 944 (commercial product A) (100 μg); 2 = Chimassorb 905, molecular mass 2174 (25 μg); 3 = Tinuvin 770, molecular mass 427 (1250 μg); 4 = THF solvent (negative peak).

Accuracy

The accuracy of the present method appears satisfactory, as shown by the average concentrations of Chimassorb 944 determined by HPLC in comparison with those evaluated by nitrogen analysis [13,14] on polypropylene samples at 0.025% and 0.15% of additive: the recovery ranges from 90 to 100% (Table I). Moreover, nitrogen analysis carried out on polymers after extraction shows that the extraction procedure is efficient as no residual additive is detected.

TABLE I

RECOVERY TESTS ON POLYPROPYLENE PELLETS BY THE HPLC METHOD

Sample	Chimassorb 944 by nitrogen analysis (%, w/w)	Chimassorb 944 by this HPLC method (%, w/w)	Recovery (%)
A	0.027	0.025	92
B	0.145	0.0150	103
C	0.138	0.140	101

Interference

As outlined above, interference from the usual antioxidants and UV stabilizers is avoided. However, each monomeric and polymeric HALS may interfere to some extent, depending on its chemical structure. For instance, Tinuvin 770 interferes with Chimassorb 944 determination at weight ratios Tinuvin 770:Chimassorb 944 $\geqslant 8$.

Detection limits

An effectively low detection limit can be achieved for either stabilizer, *i.e.*, 1.0 μg for Chimassorb 944 and 0.5 μg for Cyasorb 3346, corresponding to a concentration of 10 and 5 ppm in the polymer sample, respectively.

Precision

The repeatability of the HPLC method was evaluated on the basis of several independent runs of the same polypropylene sample, containing 0.17% of Chimassorb 944. The following results were obtained: average value (%, w/w): $\bar{X} = 0.167$. Relative standard deviation: $s/\bar{X} = 0.030$. Confidence limits (95% probability) for a single analysis (%, w/w): ± 0.012 ($\pm 7\%$).

ACKNOWLEDGEMENT

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CHROMSYMP. 2146

Analysis of polyethylene glycol and derivatives by high-performance liquid chromatography using elevated temperatures and low-wavelength ultraviolet detection, and supercritical fluid chromatography

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ABSTRACT

A gradient reversed-phase high-performance liquid chromatography system is described for the characterisation of polyethylene glycols (PEGs), methoxy polyethylene glycols (MPEGs) and methoxy polyethylene glycol methacrylates. The system enables oligomeric distributions to be obtained for these types of materials with average molecular weights of up to 4000 dalton. The requirements to achieve the necessary detection and separation efficiency have been studied including the column type, operating temperature and solvent composition.

The PEGs have been analysed by capillary supercritical fluid chromatography, using carbon dioxide as the mobile phase. The results obtained from the two chromatographic techniques have been compared and the relative merits discussed.

INTRODUCTION

The uses of polyethylene glycols (PEGs) and their derivatives are widespread and include such applications as the production of emulsifiers, surfactants, detergents, cosmetics as well as their uses in foodstuffs and wood preservation. There are therefore, many requirements for accurate material quality control and product characterisation, paticularly for environmental monitoring.

Although high-performance liquid chromatography (HPLC) is the technique of choice for the separation of ethoxylated oligomers, the subsequent detection and quantification has proved to be difficult. The insensitivity of these compounds towards conventional UV detection is due to the lack of chromaphoric structure. The use of refractive index (RI) detection [1,2], whilst having an inherent low sensitivity, also limits any method to isocratic elution and the range of component polarities which can be chromatographed.

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To overcome these problems derivatisation techniques have been used to produce dibenzoates [2], 3,5-dinitrophenylates [3] and phenyl isocyanates [4], and thereafter allow detection by UV. Berry [5] has shown that low-wavelength UV detection is applicable to the determination of compounds with a low or minimal absorptivity, providing almost universal detection. Berry also reported that at 210 nm 0–100% acetonitrile gradient elution could be used with the addition of sodium azide to balance solvent absorbances. However. at lower wavelengths solvent impurities prohibited sensitive detection.

Snyder and Van der Wal [6] have previously reported the separation of polyethylene glycol oligomers of molecular weights up to 1200 dalton, using 0–35% acetonitrile gradients with UV detection at 185 nm. Again the limiting factor being the purity of the acetonitrile, with gradient "humps" and "ghost" peaks being produced when 40% acetonitrile was exceeded.

With the advent of more pure solvents (HPLC and spectroscopic grades), the use of sodium azide for solvent absorbance equalisation at 190 nm, enables the achievement of full range gradient elution. We have found that the use of this type of system, with coupled columns and elevated temperatures (from ambient up to 80°C), has made possible the separation of PEG oligomers of molecular weights up to 5000 dalton. This technique has also been suitable for PEG derivatives such as methoxy PEGs and methoxy PEG methacrylates with molecular weights up to 2500 dalton.

The ability of supercritical fluid chromatography (SFC) to utilise flame ionisation detection (FID), makes it an attractive technique to use for the characterisation of these types of compounds. The comparison of SFC–FID and HPLC (with low-wavelength detection) made here, demonstrates the limitations and advantages of both techniques for this type of analysis.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of 2 LKB 2150 HPLC pumps with a LKB 2152 gradient controller. The samples were injected by means of a Valco C6W valve, fitted with a 20-μl loop. The detector was a Kratos Spectroflow 757, which was used with a continuous nitrogen purge of the flow cell to reduce ozone build-up. An Anachem column oven (Gilson, Luton, U.K.) was used for the elevated temperature work, and the data system was a Trivector Trilab 3000 (Vinten Analytical, U.K.).

The HPLC columns used included: (i) the cartridge columns (S-5 ODS2) supplied by Phase Separations (Clwyd, U.K.). These are 250 mm × 4.6 mm I.D. stainless-steel, packed with 5-μm octadecyl silane bonded phase material of 80 Å pore diameter; (ii) a column of the same dimensions but packed (in our laboratory) with 5-μm Hypersil WP-300 (Shandon Southern) octyl bonded phase material, which has a pore diameter of 300 Å.

The SFC system was a Lee Scientific 600 with a Lee Mk III flame ionisation detector. The column was a 10 m × 50 μm I.D. SB-octyl capillary column, also purchased from Lee Scientific. The mobile phase used was carbon dioxide (SFC grade) obtained from Air Products. The samples were prepared in dichloromethane solutions, and were injected using timed split injection over 30 ms with a 200-nl loop. All data were collected on a VG Multichrom PDP11/23 data system.

TABLE I

HPLC ANALYSIS CONDITIONS

Analyte	Gradient profile						Temparature (°C)
	Step 1		Step 2		Step 3		
	%B	Time (min)	%B	Time (min)	%B	Time (min)	
PEGs 200–3400 (Fig. 1)	25–40	15	40–55	45	55–60	45	80
PEG 2000 (Fig. 3)	35–50	40	50	20	–	–	60
PEG 4000 (Fig. 2)	45	45	45–60	90	–	–	80
MPEG 2000 (Fig. 5)	35–50	40	50	20	–	–	60
MPEG 2000 methacrylate (Fig. 4)	35–50	40	50	20	–	–	60

Reagents

The polyethylene glycol materials were purchased from BDH (Poole, U.K.) and the sodium azide from Fisons Labs. (Loughborough, U.K.), the acetonitrile (used as solvent B) was far-UV grade from Romil Chemicals (Loughborough, U.K.), and all water (used as solvent A) was purified by means of a Millipore Milli-Q system. The sodium azide was added to the water at a concentration of 5 μg/l. All solvents were continually degassed with helium, and a total flow-rate of 1 ml/min was used for all analyses.

The HPLC analysis conditions, including the gradient profiles, used for the different analytes are summarised in Table I.

RESULTS AND DISCUSSION

A number of specific procedures have been reported in the literature for the analysis of PEG oligomers. The work by Snyder [7] predicted that convex gradient profiles are the most suitable for PEG oligomeric separations. Whilst this may be a requirement for optimal resolution, the simple linear profiles used in this work were found to be adequate for the characterisation of the materials investigated. The combination of two reversed-phase columns as used here, provides a relatively large number of theoretical plates (*ca.* 40 000). This, together with the linear gradients and the elevated operating temperatures has enabled the higher-molecular-weight PEGs to be resolved into individual oligomers, albeit with an increased analysis time. Figs. 1 and 2 show the scope of the method for oligomeric separations, although to achieve the broad molecular weight range, a high sample loading (5 mg of material injected) was used. This necessitated a relatively high detector sensitivity (0.05 a.u.f.s.) which does show some baseline drift. The separation achieved for the PEG 4000 oligomers would appear to be approaching the separation and detection limits of the technique for these compounds. It was found that a relatively long (45 min) initial isocratic period was required to provide high capacity factors (k'). The resulting long column residence times are more suitable for separating high molecular weight compounds with slow mass transfer characteristics. For the analysis of a "single" material (rather than

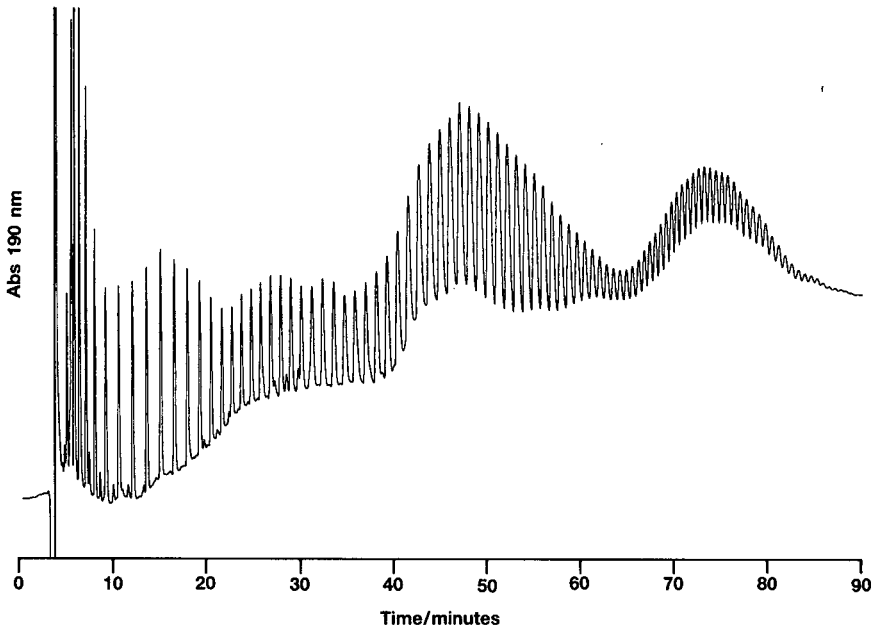

Fig. 1. HPLC analysis of a blended mixture of PEGs (with average molecular weights of 200, 400, 600, 1000, 1500, 2000 and 3400 dalton) carried out on two ODS columns at 80°C and with a detector sensitivity of 0.05 a.u.f.s.

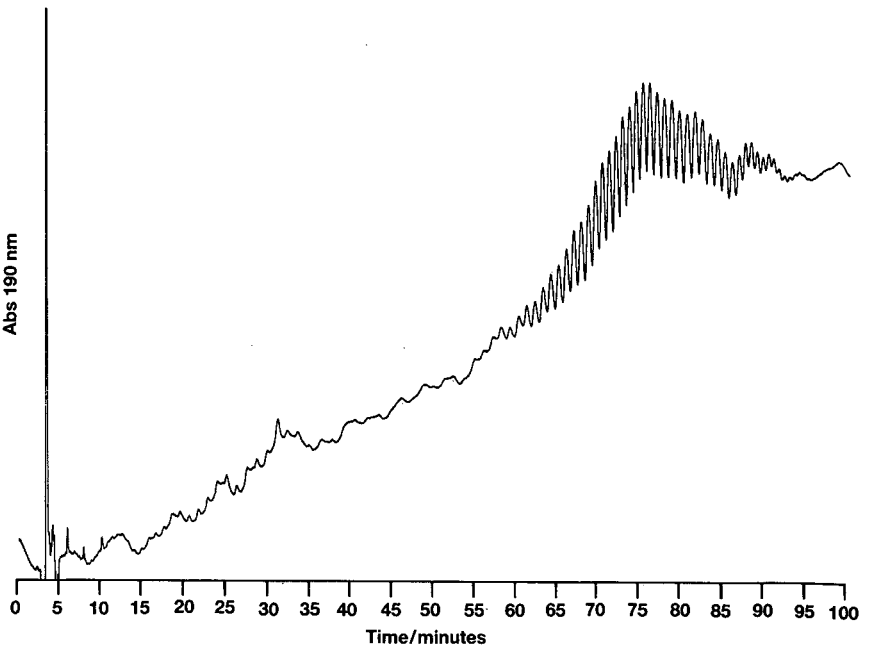

Fig. 2. HPLC analysis of PEG with an average molecular weight of 4000 dalton. The analysis conditions are given in Fig. 1 and Table I.

a blend), a sample size of 1 mg and a detector sensitivity of 0.2 a.u.f.s. provided adequate sensitivity with minimum baseline drift (see Figs. 3–5).

The problems associated with low-wavelength UV detection such as solvent background absorbance and baseline drift, have been documented [5–7]. The minimum useable detection wavelength for current commercial deuterium lamps appears to be 185 nm. Although the sensitivity obtained at this wavelength was significantly better than that at 190 nm, the apparent absorbance of the sodium azide is considerably reduced. This effectively nullifies the solvent absorbance equalisation by the azide and the baseline follows the profile of the gradient used. The use of nitric acid in place of the azide for detection of PEGs at 185 nm has been described [8] and this may provide a more universal detection capability. However, the problems associated with solvent impurities are exacerbated by detection at 185 nm. This has often limited the amount of organic modifier which can be used, and therefore a less polar stationary phase must be selected.

For the system described here, it was found that 100% acetonitrile could be used, if necessary, with adequate sensitivity at 190 nm. However, a maximum of 60% acetonitrile, for the PEGs (Fig. 2) and 50% for the PEG derivatives (Figs. 4 and 5), was all that was required to elute the oligomers up to 4500 dalton. The separations shown here were found to be reproducible, and no interference from "ghost" peaks or "humps" from the gradient profiles were obtained. The background absorbance levels were found to vary with different solvent batches, and hence some baseline drift was obtained without individual solvent absorbance equilisation for each batch.

Gel permeation chromatography (GPC) has also been used, together with

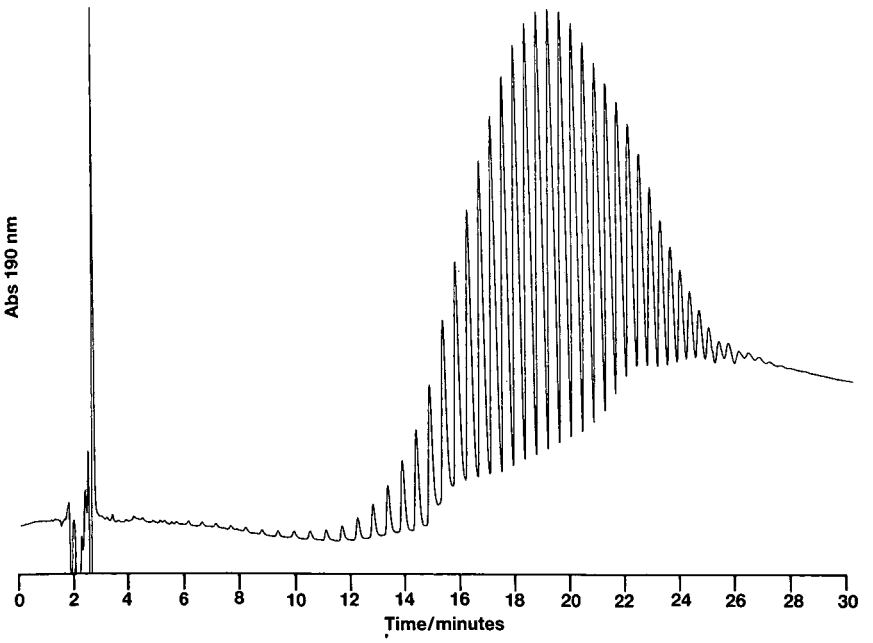

Fig. 3. HPLC analysis of PEG with an average molecular weight of 2000 dalton, carried out on two ODS columns at 60°C and with a detector sensitivity of 0.2 a.u.f.s.

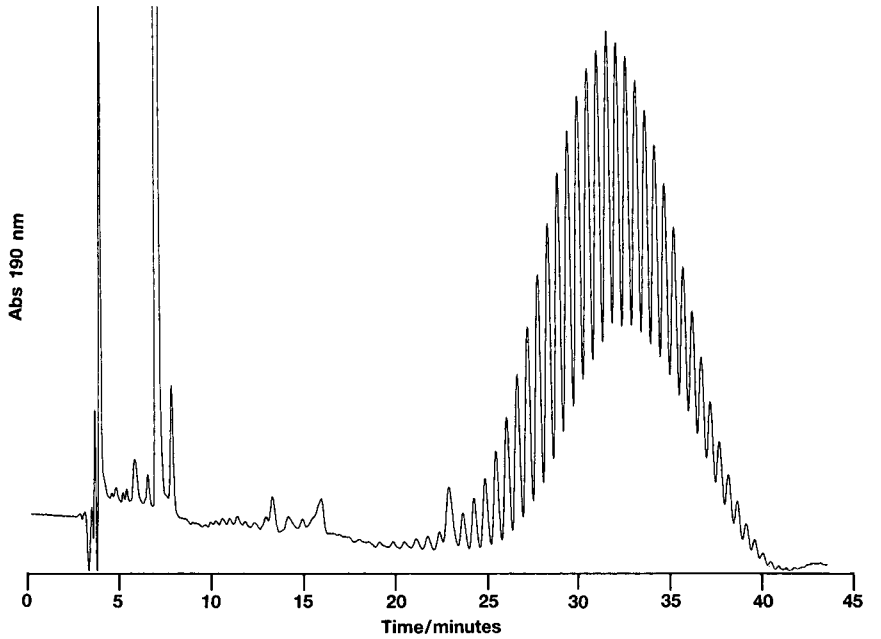

Fig. 4. HPLC analysis of methoxy PEG methacrylate with an average molecular weight of 2000 dalton. The analysis conditions are given in Fig. 3 and Table I.

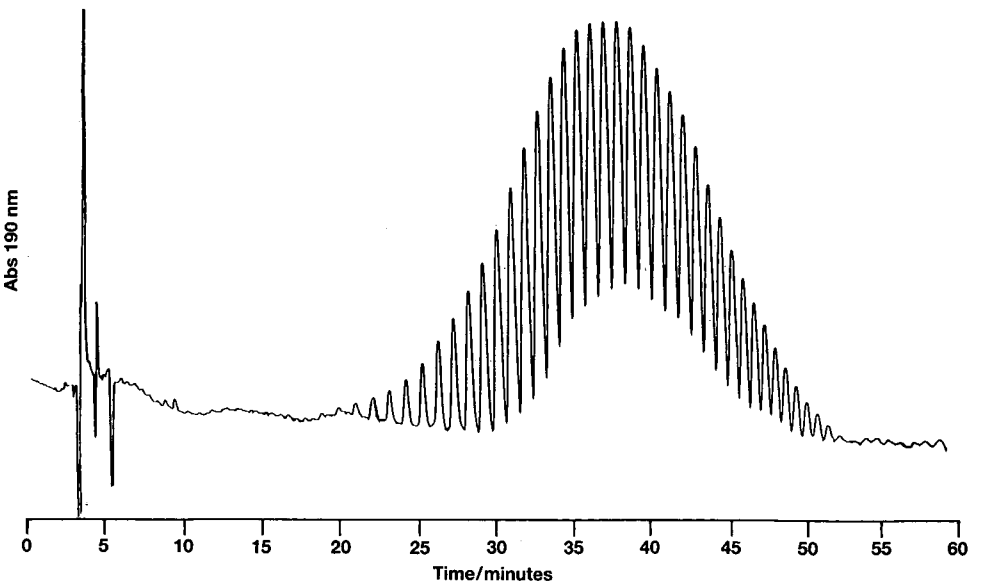

Fig. 5. HPLC analysis of methoxy PEG with an average molecular weight of 2000 dalton, carried out on an ODS column coupled with a 300 Å octyl column at 60°C, and with a detector sensitivity of 0.2 a.u.f.s.

low-wavelength UV detection, for the analysis of PEGs [9]. GPC alone was not able to separate the oligomers of PEGs >2000 dalton, and a mixed mode separation mechanism of GPC and adsorption was postulated with a combination of 100 Å and 300 Å columns. The pore size of the column packing does not appear to have a large effect on the separation obtained by adsorption chromatography. As a part of this work, the results obtained from the analysis of PEG 2000, MPEG 2000 and MPEG 2000 methacrylate achieved on two 80 Å ODS columns (Fig. 3), were not significantly different from those obtained from an 80 Å ODS coupled with a 300 Å octyl column (Fig. 5). Thus it would appear that at elevated temperatures, the large PEG molecules have suitable mass transfer characteristics for interaction with conventional (80 Å pore diameter) bonded phase HPLC columns.

Temperature effects

The operating temperature can have a profound effect on chromatographic resolution. This is due to the temperature dependence of the phase capacity ratio k', which is usually expressed in terms of the ratio of the solute concentration in stationary (C_s) and mobile (C_m) phases:

$$k' = \frac{C_s V_s}{C_m V_m} \tag{1}$$

where V_s and V_m are the volumes of stationary and mobile phases.

The exponential dependence of k' on temperature (T) is such that changes in operating temperature can have a significant effect on component retention and resolution:

$$\ln k' \propto \frac{\Delta H}{RT} \tag{2}$$

where ΔH is the standard enthalpy of sorption and R is the molar gas constant.

As values of ΔH are usually negative, an increase in temperature will result in a decrease in k', *i.e.*, C_m increases producing shorter retention times. A sequence of HPLC analyses for PEG 2000 spiked with four probe compounds, with increasing operating temperature is shown in Fig. 6. The probe compounds (benzamide, phenol, *p*-cresol and anisole) behave in a typical manner with retention decreasing with a rise in temperature. However, the reverse is observed with the PEG 2000 components, the resolution and retention being significantly increased with 20°C increments of temperature. It is clear that the simple equation above (eqn. 2) cannot be applied to PEG materials directly.

The irregular retention behaviour of relatively short chain ethoxylated oligomers has been studied in detail by Melander *et al.* [10], and it has been shown [11,12] that these oligomers can exist in at least two conformational forms. One of these is a compact dihedral helical structure (called the meander form) and the other is an extended open coil (called the zigzag form), each having intrinsically different retention characteristics. An equilibrium exists between these forms which is affected by several factors: (i) the ethoxylate chain length; (ii) temperature; and (iii) solvent composition.

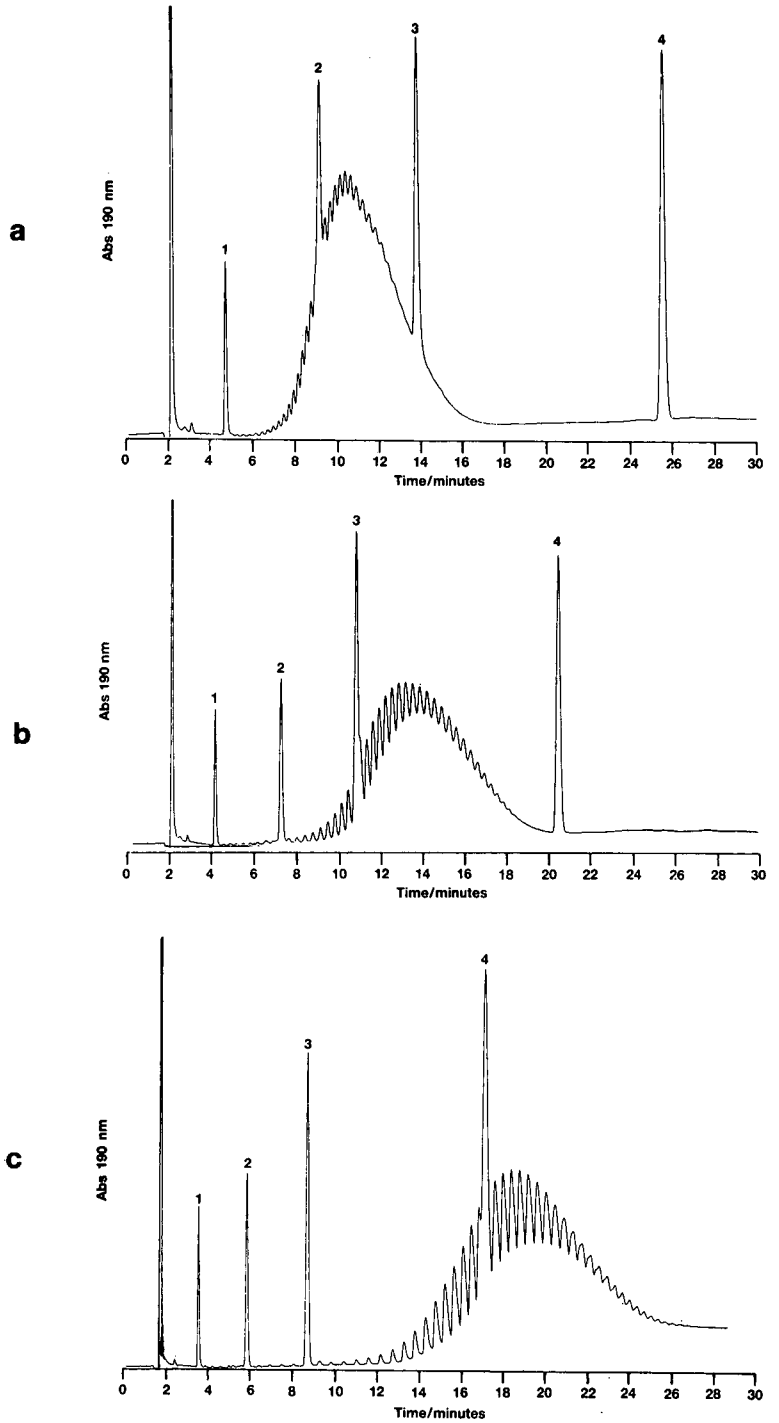

Fig. 6. HPLC analysis of PEG with an average molecular weight of 2000 dalton, spiked with a probe compound mixture: 1 = benzamide; 2 = phenol; 3 = *p*-cresol and 4 = anisole. The two ODS columns were used at (a) ambient temperature, (b) 40°C and (c) 60°C.

The most significant effect, for the analyses of the PEGs and PEG derivatives described here, was that from the column operating temperature. At elevated temperatures the equilibrium is in favour of the zigzag conformation, which has a larger molecular surface area than the corresponding meander form. The work by Melander and co-workers [10,13] has shown that retention factors, observed in reversed-phase chromatography, will increase with increasing molecular surface area, provided all other conditions are identical. The increased resolution obtained is likely to be due to two factors: (i) the higher effective column efficiencies at higher temperatures, which are realised by the decreased viscosities and increased solute diffusion coefficients [14], and (ii) the better interaction of the linear zigzag conformation with the linear alkyl groups of the bonded phases.

Analysis by SFC

For this work a 10 m × 50 μm I.D. SB-octyl capillary column was used, which provided approximately 60 000 theoretical plates. The chromatograph was operated at a temperature of 100°C with carbon dioxide as the mobile phase, and with a pressure programme from 100 to 400 bar over 30 min. The results obtained showed good resolution for the lower molecular weight PEG oligomers, and PEG 200, 400 and 600 (see Fig. 7) could be fully characterised. The analysis times were significantly less, *e.g.*, 30 min for PEG 600 by SFC, compared to 50 min by HPLC. However, for PEGs with average molecular weights greater than 600, the resolution obtained decreased rapidly. This is illustrated by the results obtained for a blended mixture of PEG 200, 400, 600 and 800 (see Fig. 8). The later eluting higher-molecular-weight oligomers are detected as a very broad unresolved envelope. This could be due in part to the poor solubility of the higher-molecular-weight oligomers in carbon dioxide. Modifiers, such as methanol, could be added to the carbon dioxide to improve the solubility, but the choice and concentration of modifier is somewhat limited by FID.

CONCLUSIONS

The present quality of commercial HPLC and spectroscopic-grade solvents, and

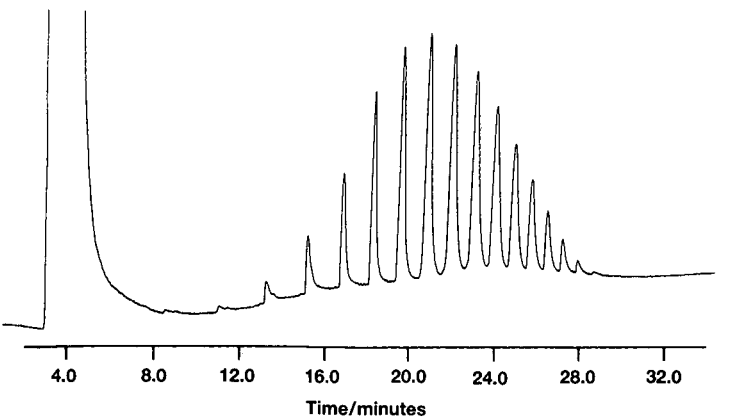

Fig. 7. SFC analysis of PEG with an average molecular weight of 600 dalton.

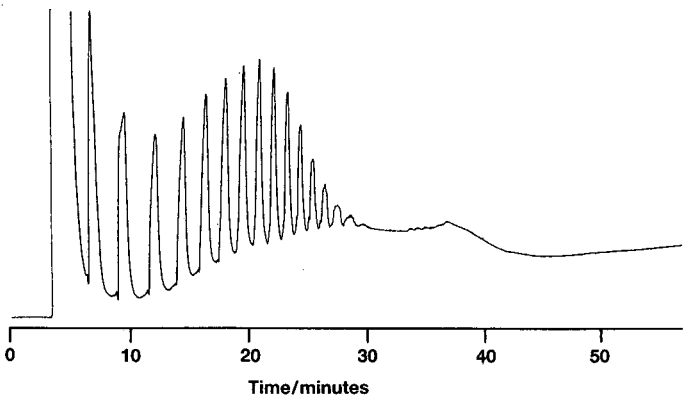

Fig. 8. SFC analysis of a blended mixture of PEGs with average molecular weights of 200, 400, 600 and 1000 dalton.

UV detection systems enables sensitive, full range acetonitrile–water gradient elution to be achieved, with a detection wavelength of 190 nm.

HPLC with low-wavelength detection can be used to characterise polyethylene glycols, methoxy polyethylene glycols and methoxy polyethylene glycol methacrylates in terms of their oligomeric distribution. The system developed has enabled materials containing oligomers of 4500 dalton to be analysed.

For low-molecular-weight materials (<800 dalton), SFC–FID is the preferred technique as it provides a more straighforward procedure and shorter analysis times.

The use of coupled 80 Å, 5-μm reversed-phase columns at elevated temperatures, provides the necessary separation media for these compounds without the use of wide pore or GPC stationary phases. It is postulated that the linear zigzag conformation of ethoxylated materials (which is induced at higher temperatures) can interact more effectively than the meander form, with conventional bonded reversed-phase materials.

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Micro liquid chromatography with fluorescence detection of thiols and disulphides

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ABSTRACT

Several biological thiols (cysteine, homocysteine and glutathione) together with their disulphides (cystine, homocystine and oxidized glutathione) could be simultaneously determined by micro liquid chromatography (LC) with fluorescence detection. Based on an existing procedure, the samples were first treated with the thiol-specific fluorigenic reagent ABD-F followed by disulphide reduction with tributylphosphine and SBD-F derivatization of the reduced disulphides. Different mobile phases, columns, injection volumes and detection systems were tried for the optimization of the separation. Quantitative studies gave detection limits at the picogram level per 60-nl injection volumes. Calibration graphs were linear with correlation coefficients >0.999. The possibility of applying gradient elution to this system represents an advantage over high-performance thin-layer chromatographic assays. Likewise, the advantages of micro-LC over classical high-performance liquid chromatography may contribute to the choice of the most suitable system depending on the analytical requirements.

INTRODUCTION

The identification and determination of biological thiols and disulphides in living organisms is of considerable importance for assessing the normal functioning of cell metabolism. Certain biochemical disorders may be detected by observing the altered thiol-to-disulphide ratio. Amongst these are cystinuria, homocystinuria and haemolytic anaemia due to glutathione synthetase deficiency [1,2].

Numerous methods for the selective and sensitive determination of the highly reactive thiol group have been previously described. Most are based on high-performance liquid chromatography (HPLC) with UV absorption, electrochemical or fluo-

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rescence detection [3–5]. However, there have been fewer studies on the simultaneous determination of oxidized and reduced thiols. Traditionally, these assays were performed by reducing the disulphides to the corresponding thiols with a suitable reducing agent prior to their detection. The concentration of disulphide was then calculated by subtraction from the total thiol concentration (thiol and disulphide) [6,7]. Other methods include the determination of GSH and cysteine together with their disulphides by HPLC and dual electrochemical detection [8,9]. However, these studies did not consider the specific behaviour of homocysteine, the essential biological amino acid, a homologue of cysteine. Toyo'oka *et al.* [10] reported on a simultaneous determination method for thiols and disulphides based on their selective derivatization with two fluorogenic labelling reagents, ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate (SBD-F) and 4-(aminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) (Fig. 1). They described the optimum reaction conditions and the main physicochemical properties of the reagents and the derivatives [11]. The method was then succesfully applied to the analysis of rat and hamster tissues [12].

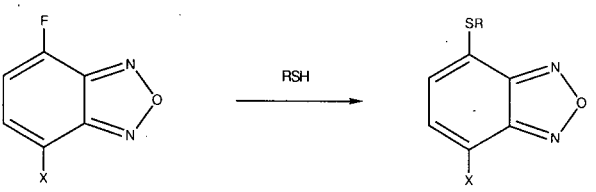
This paper is based on the selective thiol and disulphide derivatization procedure optimized by Toyo'oka *et al.* [10], applied to the recently introduced and rapidly growing field of micro-LC. Amongst the main advantages of this technique over conventional HPLC are the low consumption of mobile phase, stationary phase and sample, apart from the better permeability and efficiency of the columns [13]. As described in a previous paper [14], conventional HPLC equipment could be adapted to micro-LC analysis by using a split flow system in combination with a laboratory-made square quartz cell for fluorescence measurements. In this work, the latter system was optimized further for the simultaneous determination of thiols together with their disulphides.

EXPERIMENTAL

Chemicals

Glutathione (GSH) and cystine were obtained from Merck (Darmstadt, Germany), cysteine and homocystine from Sigma (St. Louis, MO, U.S.A.) and homocysteine and oxidized glutathione (GSSG) from Aldrich (Beerse, Belgium). All compounds were chemically pure and used as received.

The fluorigenic derivatizing reagents SBD-F and ABD-F were obtained from Wako (Neuss, Germany). Tributylphosphine (TBP), used for reducing the disulphides, was dissolved in dimethylacetamide (DMA), both from Janssen (Beerse, Bel-



ABD-F : $X=SO_2NH_2$

SBD-F : $X=SO_3^-NH_4^+$

FLUOROPHORES

Fig. 1. Structures of ABD-F and SBD-F and general reaction with thiols (RSH).

gium). Acetonitrile and water for the mobile phase were of HPLC grade (Alltech, Deerfield, IL, U.S.A.). Disodium EDTA (Merck) was added to all thiol and reagent solutions at a concentration of 2.0 m*M* to prevent metal-catalysed thiol oxidation.

Derivatization reaction

The present sample preparation method was initially proposed by Toyo'oka *et al.* [10]. Standard thiol and disulphide solutions were prepared by mixing equal volumes of each thiol (cysteine, homocysteine and GSH) and each disulphide solution (cystine, homocystine and GSSG), all compounds dissolved in 0.1 *M* aqueous sodium borate buffer (pH 9.5) containing 2.0 m*M* disodium EDTA. To a 1-ml aliquot of this mixture was then added an equal volume of the first fluorogenic reagent, ABD-F (1.0 m*M* dissolved in the above-mentioned borate buffer solution). The reaction mixture was vortex mixed, heated in a water-bath at 60°C for 5 min and cooled in ice. The excess of unreacted ABD-F was extracted by the addition of 4.0 ml of ethyl acetate, followed by vigorously shaking for 1 min and centrifuging at 1850 *g* for 5 min. To 400 μl of the lower aqueous layer were added 550 μl of SBD-F (1.0 m*M* dissolved in the previously mentioned 0.1 *M* sodium borate buffer solution) and 50 μl of the reducing agent TBP, 10% (v/v) in DMA. This final reaction mixture was heated at 60°C for 20 min, cooled in ice and subjected to micro-LC analysis with fluorescence detection at room temperature.

Apparatus and chromatography

Conventional HPLC equipment was adapted to micro-LC studies by connecting the pump (Model 5560 gradient elution delivery system; Varian, Walnut Creek, CA, U.S.A.) to a T-flow-split system (Valco, Houston, TX, U.S.A.), which divides the mobile phase between the micro-LC column (250 × 0.32 mm I.D. fused-silica capillary filled with 5-μm RoSiL C_{18}; Bio-Rad RSL, Eke, Belgium) and a by-pass conventional HPLC column (5-μm RoSiL C_{18}, 150 × 4.6 mm I.D.). An internal volume injector (60–1000 nl, Valco CI4W) and a 10-μl Valco external injector loop (CV6U) was used for the micro-LC system. The optimized mobile phase conditions were gradient elution with 0.15 *M* H_3PO_4 (A)–acetonitrile (B) from 92:8 to 70:30 (v/v) in 15 min followed by isocratic elution with A–B (70:30, v/v) for 5 min. Chromatography was carried out at room temperature at a flow-rate of 3.0 μl/min, which was frequently checked by connecting an empty 10-μl syringe to the column end and timing the advance of the liquid meniscus. The chromatographed samples were detected by means of a fluorescence detector (Shimadzu Model RF-535; Pleuger, Wijnegem, Belgium) at λ_{exc} = 380 nm, λ_{em} = 510 nm, into which a laboratory-made 312-nl square quartz cell was inserted (0.25 × 0.25 × 5.0 mm). For comparative purposes, a Model 272 UV detector (Wescan, Santa Clara, CA, U.S.A.) containing a similar laboratory-made square quartz cell was used. For recording the signals, a Model 2020-000 recorder, (Linear Instruments, Reno, NV, U.S.A.) was employed. Integration was performed on a Chromatopac C-R3A integrator system (Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

Optimization of the micro-LC chromatographic system

Mobile and stationary phases. Based on the chromatographic separation of a mixture of several SBD- and ABD-labelled thiol derivatives [14], the following gradient eluent was initially tried: 0.15 *M* H_3PO_4 (A)–acetonitrile (B), from 95:5 to 70:30 (v/v) in 15 min followed by isocratic elution with A–B (70:30, v/v) for 5 min at a flow-rate of 3.0 μl/min. This resulted in an optimum separation of the three ABD derivatives corresponding to the thiols cysteine, homocysteine and GSH. The disulphide compounds, which had initially been reduced to the corresponding thiols with TBP and derivatized with SBD-F, eluted later and overlapped under the above conditions. Therefore, less acetonitrile in the gradient composition was subsequently tested followed by a higher initial H_3PO_4 content in the eluent. The final optimized eluent conditions were a gradient of A–B from 92:8 to 70:30 (v/v) in 15 min followed by isocratic elution with A–B (70:30, v/v) for 5 min. The resulting chromatogram is shown in Fig. 2. The SBD derivatives eluted earlier than the ABD derivatives, although the order of elution was the same within one group of thiols (*i.e.*, cysteine first followed by homocysteine and GSH), probably owing to the increasing hydrophobic-

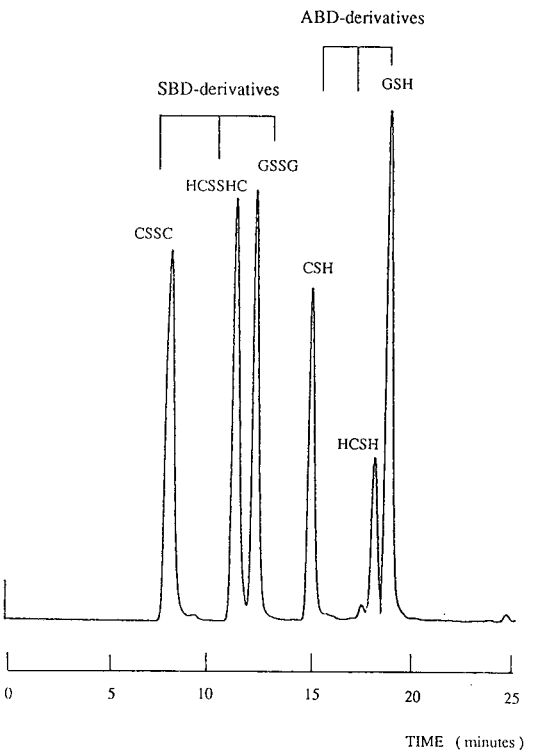


Fig. 2. Chromatogram obtained from micro-LC with fluorescence detection of 60 nl of a mixture containing CSH (cysteine) (125 μg/ml), HCSH (homocysteine) (13 μg/ml), GSH (glutathione) (45 μg/ml), CSSC (cystine) (180 μg/ml), HCSSHC (homocystine) (29 μg/ml) and GSSG (oxidized glutathione) (68 μg/ml).

ity and dimensions of the compounds. Even though the retention times of homocysteine and GSH were similar in both types of derivatives, a better separation of the four compounds in one run either by inclusion of less H_3PO_4 or by increasing the acetonitrile content was not feasible, as the SBD and the ABD derivatives overlapped, respectively. The eluent indicated above gave the most acceptable results.

Fused-silica capillaries (250 × 0.32 mm I.D.) packed with 5-μm C_{18} silica gel were used throughout. Smaller particle sizes did not provide significant changes in the chromatographic separation. The application of longer columns (1 m), described previously [14], resulted in better resolution but required longer analysis times.

Injection volume. Once the column and mobile phase had been optimized, different injection volumes were tried in an attempt to increase the sensivity without a significant decrease in efficiency. Injection volumes of 60, 200, 500, 1000 and 10000 nl were tried, giving in all instance an acceptable separation of the derivatives owing to the "on-column focusing" effect [15] and the use of gradient elution. This allows the assay of samples prepared for conventional HPLC on micro-LC systems. However, the first-eluting peak (cysteine-SBD) suffered considerably from high injection volumes as it was severely broadened and therefore decreased the overall resolution of the separation. Injection volumes of 60 nl were therefore preferred and used throughout.

Detection system. Fluorescence detection was preferred to UV absorption detection because of the higher sensitivity and selectivity. UV absorption measurements using a similar laboratory-made square quartz cell gave a considerable baseline drift owing to refractive index changes in the gradient elution system and also a noisy background signal. As much as a 10-fold increase in the detection limits was observed on going from fluorescence to UV measurements. "On-column" fluorescence detection [14] was tried previously but did not improve the results.

Qualitative and quantitative separation of thiols and disulphides

The chromatogram in Fig. 2 shows the optimized simultaneous separation of the three thiols cysteine, homocysteine and GSH as their ABD derivatives together with their respective disulphides cystine, homocystine and GSSG as their SBD deriv-

TABLE I

RETENTION TIMES AND DETECTION LIMITS OF THE VARIOUS THIOLS (DETECTED AS ABD DERIVATIVES) AND DISULPHIDES (DETECTED AS SBD DERIVATIVES)

Compound	Retention time (min)	Detection limit (pg per 60 nl injection)
Thiols		
Cysteine	16.3	83
Homocysteine	19.7	18
GSH	20.5	20
Disulphides		
Cystine	8.7	108
Homocystine	12.2	15
GSSG	13.3	35

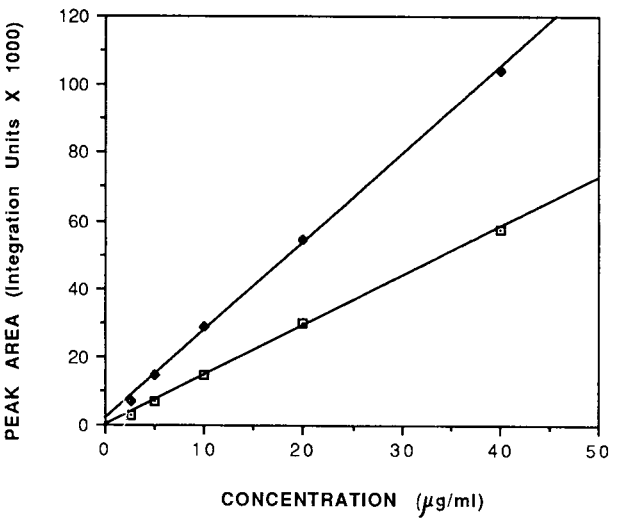


Fig. 3. Calibration graphs for (◆) GSH and (□) GSSG. GSH: $y = 1.8962 + 2.5702x$; $R^2 = 0.999$. GSSG: $y = -0.18450 + 1.4523x$; $R^2 = 0.999$.

atives. Their retention times and detection limits (signal-to-noise ratio >2) are given in Table I. The relative standard deviations were 0.690% and 0.810% for the retention time and peak areas, respectively (for $n = 10$, GSH-SBD). It may be concluded that, in general, the ABD derivatives gave higher fluorescence signals than the corresponding SBD derivatives (note that the signals of the SBD derivatives should correspond to more or less double the respective signals of the thiol derivatives). The relatively poor detection limit observed for cysteine-ABD is probably a result of its low stability under the conditions of measurement and the shifts of its luminescence parameters [10]. Likewise, the detection limit of cystine was poor, in addition to its limited solubility in borate buffer media, which made heating and stirring necessary for complete dissolution. Calibration graphs (fluorescence intensity signals *versus* thiol or disulphide concentration) for GSH and GSSG as representative thiol derivatives were linear in the range 0–40 μg/ml (Fig. 3), with correlation coefficients >0.999. Blank runs (containing no thiol compound) gave no peaks under the same chromatographic conditions.

Comparison with other chromatographic systems

In a previous paper [16] the applied derivatization procedure was similarly performed in high-performance thin-layer chromatographic experiments, and GSH and GSSG could be adequately separated and determined. However, cysteine, homocysteine and their disulphides could not be simultaneously separated as the R_F values of their derivatives were too close for reliable quantification.

The advantage of the present micro-LC system lies in its versatility. The possibility of applying gradient elution to the system allowed the selective separation of all the thiols and disulphides simultaneously in one run. In this instance the detection limits of GSH and GSSG were similar in both chromatographic systems.

In conventional HPLC [10], the detection limits achieved were slightly better for the derivatives assayed, although the previously mentioned advantages of micro-LC [13] may be of considerable relevance whenever a chromatographic system is to be chosen for a particular purpose.

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CHROMSYMP. 2328

Quantitative structure-type analysis of heterocompound–hydrocarbon mixtures

VI. Characterization of the nature of neutral heterocompounds in a product of brown coal refining

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ABSTRACT

Neutral heterocompounds are one of the major fractions of the pyrolysis or hydrogenation products of brown coals. They are classified into two groups by chromatographic methods. The first group (thiophenes, aliphatic ketones) elutes from the normal phase with non-polar eluents, in analogy with aromatic hydrocarbons. They can be separated from aromatic hydrocarbons by complex formation with palladium chloride [*Fuel*, 65 (1986) 270–273; *J. Chromatogr.*, 509 (1990) 27–32]. The second group elutes from the normal phase with polar eluents. The neutral heterocompounds in an extract of heat-pretreated brown coal were separated by semipreparative high-performance liquid chromatography. They were fractionated by increasing polarity and subsequently characterized by Fourier transform infrared spectrometry, protonnuclear magnetic resonance and mass spectrometry. Results fo these spectrometric methods (*i.e.* the existence of hydroxy and carbonyl residues) could be verified by derivatization with 2,4-dinitrophenylhydrazine and 3,5-dinitrobenzoylchloride followed by qualification of these derivatives by reversed-phase chromatography. The neutral heterocompounds were hydrogenated in two steps, and the resulting hydrocarbons were characterized by semipreparative and analytical high-performance liquid chromatography, as well as by capillary gas chromatography. General conclusions about the structures, as result of the combination of the different applied methods, could be made.

INTRODUCTION

The refining of brown coals by thermal or hydrogenating processes leads to products such as tars and hydrogenation products. In addition to extracts of heat-pretreated brown coals, such products include hydrocarbons (saturated and aromatic) and heterocompounds (acidic, basic and a significant fraction of neutral compounds).

The characterization of the composition of such refined and pretreated brown coals is necessary for the elucidation of the reaction mechanisms that occur during the processing. The development of an on-line semipreparative high-performance liquid

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chromatography (HPLC) method for the structure-type analysis of heterocompound–hydrocarbon mixtures and its application to the characterization of technical products of thermal and hydrogenated refined brown coals and to mineral oil processing and the reactions taking place during these processes have reported in previous papers [1–4]. The chromatographic properties of the neutral heterocompounds (NHC) are determined by their polarity. The less polar NHC (thiophenes, alkanones) elute from precolumn 1, packed with a normal-phase silica support, together with aromatic hydrocarbons, with non-polar eluents (*n*-hexane, *n*-pentane). They are separated by complex formation of the NHC on palladium chloride [5] on precolumn 2. This on-line separation (Fig. 1) has been described by Zobel *et al.* [6]. The NHC with higher polarity elute from precolumn 1 with mixtures of polar and non-polar aprotic eluents or polar aprotic eluents. These compounds are either thermally unstable or have an increased boiling point. It is not possible to characterize the individual compounds by capillary gas chromatography (cGC) coupled with mass spectrometry (MS). Spectrometric methods for the characterization of the NHC provide only circumstantial evidence for the existence of functional or structural groups.

EXPERIMENTAL AND RESULTS

Separation and characterization of NHC in extracts of heat-treated brown coal

Separation and structure-type analysis in the extract. The structure-type composition of the extract of heat-treated brown coal (referred to as sample 1) was determined by semipreparative HPLC (Fig. 1) by the method of Zobel and co-workers [1–4,6].

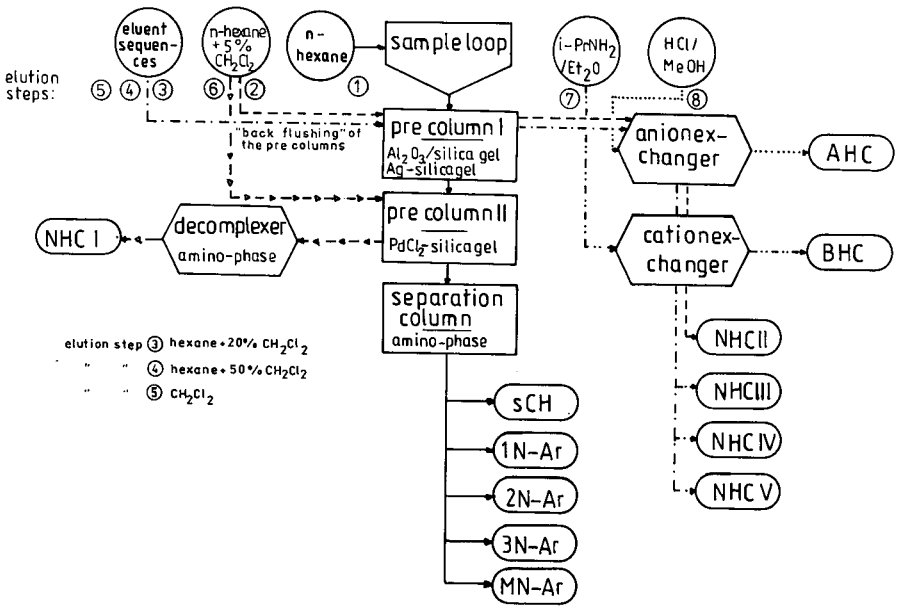

Fig. 1. Scheme of the structure-type separation.

TABLE I

STRUCTURE-TYPE CHARACTERISTICS OF AN EXTRACT OF HEAT-PRETREATED BROWN COAL

Structure type	Content (%)	Qualitative composition
sCh	24.9	Paraffins, olefines
1 N-Ar	5.9	Alkylbenzenes, tetralenes/indanes
2 N-Ar	5.3	Naphthalenes, acenaphthenes, diphenyls, fluorenes
3 N+MN-Ar	3.7	Antracenes, phenathrenes, pyrenes, chrysenes, polycyclic aromatics
NHC I	10.3	Homologous series of alkanones, dibenzothiophenes
NHC II	3.9	
NHC II	4.2	
NHC IV	8.8	
NHC V	18.3	
BHC	2.3	
AHC	8.4	

Backflushing of precolumn 1 was performed with the following eluent sequences: *n*-heptane–dichloromethane 9:1 (NHC II), *n*-pentane–dichloromethane 8:2 (NHC III), *n*-pentane–dichloromethane (NHC IV) and dichloromethane (NHC V). The structure-type composition which was found in sample 1 is listed in Table I. The following abbreviations are used in the table and throughout the whole paper: sCH, saturated hydrocarbons; *x* N-Ar cyclic hydrocarbons, where *x* indicates the number of ring systems; BHC, basic heterocompounds; and AHC, acidic heterocompounds.

The qualitative composition of the hydrocarbons and NHC I fractions was deduced from the HPLC (Fig. 2A and B) and the cGC–MS profiles. The fractions NHC I–NHC V were characterized by Fourier transform infrared (FT-IR) spectrometry, proton nuclear magnetic resonance (^{1}H-NMR) and MS.

Interpretation of the FT-IR spectra. The FT-IR spectra of the NHC II and NHC V fractions suggest the existence of OH residues (band at 3450 cm^{-1}) and C=O residues (band at 1700 cm^{-1}) in all NHC fractions and C–O–C residues (band at 1700 cm^{-1}). In the NHC I fraction an intensive band at 2930 cm^{-1} was found, suggesting aliphatic CH_2 residues. This band was very weak in the NHC V fraction (Fig. 3). The intensities of the bands at 1600 and 3080 cm^{-1} increased with the polarity of the NHC.

Interpretation of the ^{1}H-NMR spectra. From the ^{1}H-NMR spectra, the characteristics of H_{ar} (hydrogen in aromatic structures), H_α (hydrogen in the α-methyl group on aromatic rings), H_β (hydrogen in the β-methylene group of aliphatic residues and naphthenic methylene groups), as well as H_e (hydrogen in the terminal methyl group of aliphatic residues) could be deduced (Table II).

Interpretation of the mass spectra. Electron ionization mass spectra of the NHC I and NHC V fractions by 70 eV are shown in Fig. 4a and b. Fragments of aliphatic chains were found in both mass spectra (mass numbers 29, 43, 57, 71, 85, 113 and 127; see also ref. 7). In the spectrum of the NHC I fraction larger alkyl chains dominate. The fragment with mass number 18 in the NHC V fraction shows the existence of OH residues on aliphatic or naphthenic structures. Fragments of aromatic structures

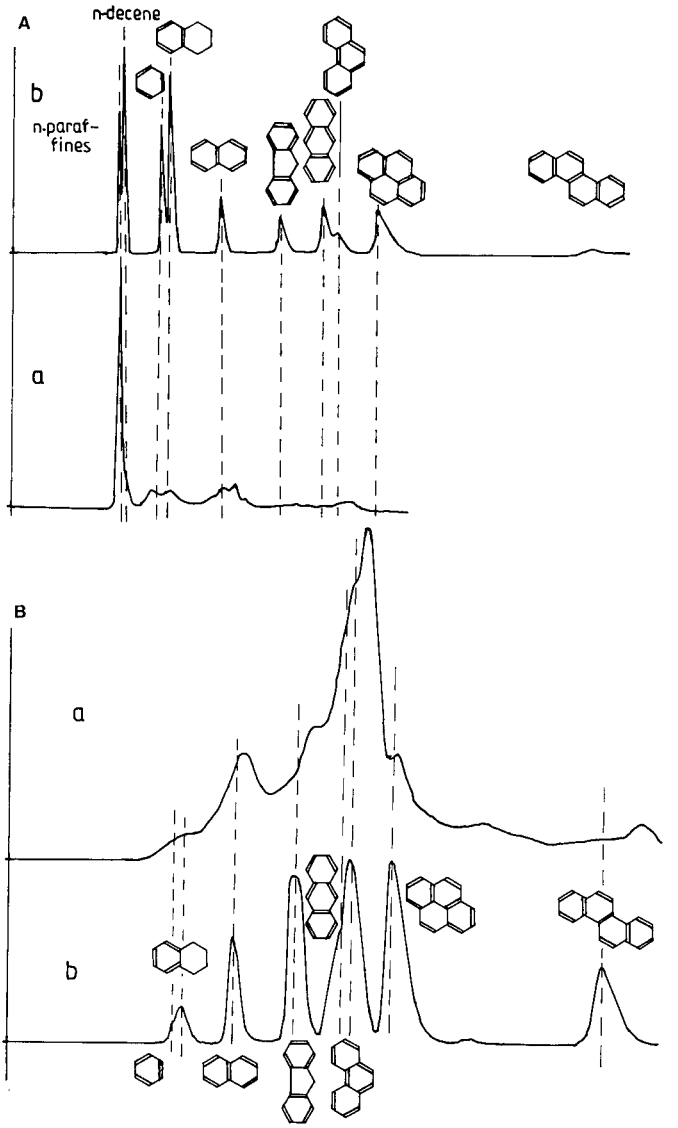

Fig. 2. (A) HPLC profiles of the hydrocarbons of the extract of the heat-pretreated brown coal (sample 1; a) and of a test mixture (b). Columns: Separon-SIX-NH_2 (150 × 3 mm I.D.) + Separon-SIX-CN (150 × 3 mm I.D.); 0.4 ml/min *n*-hexane; differential refractometer. (B) HPLC profiles of the hydrocarbons of the extract of the heat-pretreated brown coal (sample 1; a) and of a test mixture (b). Column: Separon-SIX-NH_2 (150 × 3 mm I.D.) + Separon-SIX-CN (150 × 3 mm I.D.); 0.4 ml/min *n*-hexane; UV detector 254 nm.

dominate in the NHC V fraction (mass numbers 39, 51, 65, 77, 91 and 105). In both fractions mass numbers 45, 59, 77 and 91 were found, suggesting the existence of ether groups in the compounds in these fractions, which has to be verified by other methods.

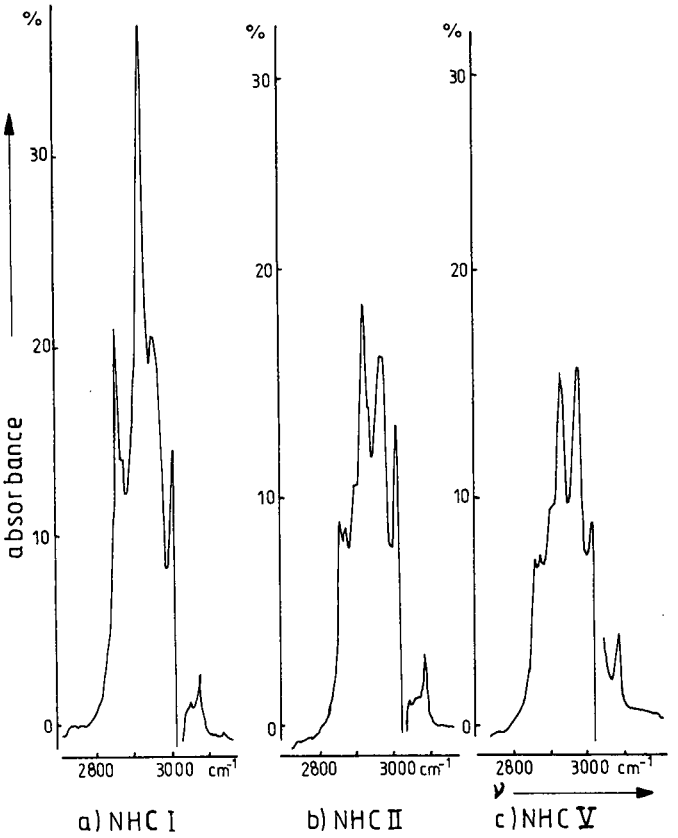

Fig. 3. FT-IR spectra of the NHC fractions.

Detection of functional groups by HPLC of derivatives. The NHC of the NHC III and NHC IV fractions were derivatized with 2,4-dinitrophenylhydrazine [8] and 3,5-dinitrobenzoylchloride [9]. The derivatives of both reactions were separated by HPLC on a Vertex–LiChrosorb RP-18 column (250 × 4 mm I.D.) with a mixture of

TABLE II

SPECTRAL CHARACTERICS OF NHC

The aliphatic character of the compounds decreases with polarity.

Fraction	Hydrogen content (%)			
	H_{ar}	H_{α}	H_{β}	H_{e}
NHC I	1.70	3.80	67.87	26.60
NHC II	5.56	16.30	58.17	20.13
NHC III	9.20	27.30	49.02	16.20
NHC IV	12.00	22.76	45.82	19.43
NHC V	13.45	32.09	43.43	11.01

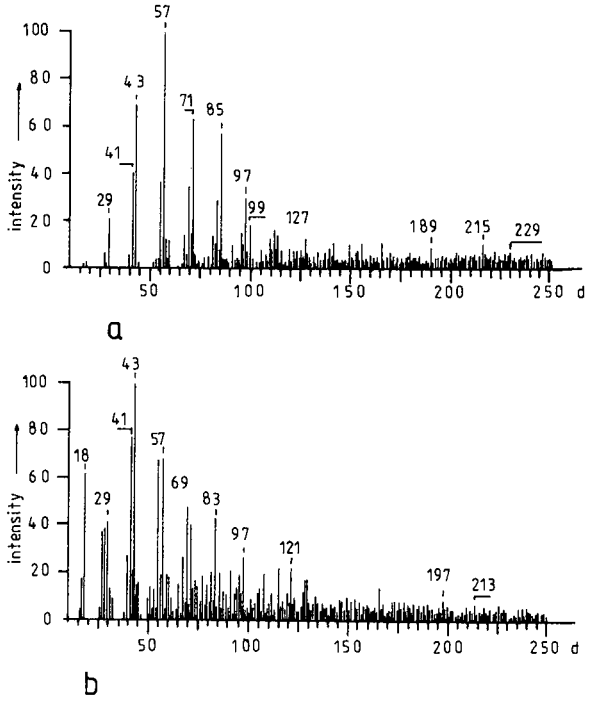

Fig. 4. Mass spectra of the NHC I (a) and NHC V (b) fractions.

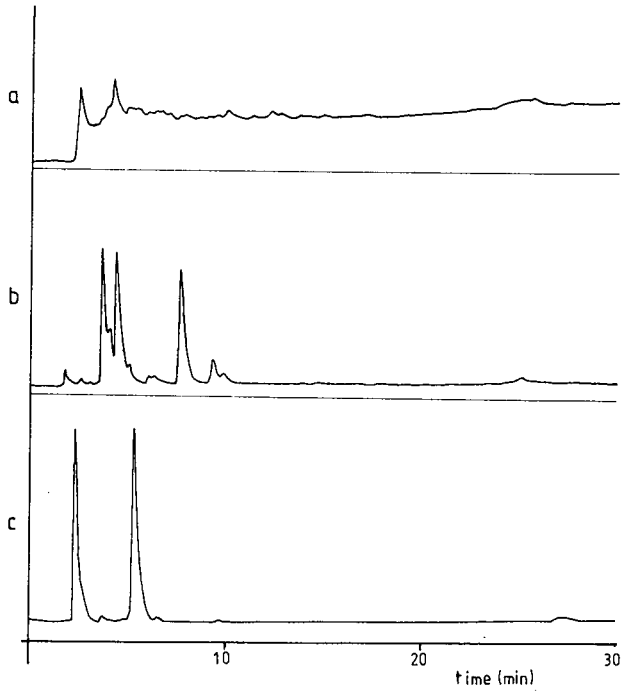

Fig. 5. HPLC profiles of the NHC III fraction (a), its 2,4-dinitrophenylhydrazones (b) and its 3,5-dinitrobenzoates. Column: LiChrosphere 100-RP-18 (5 μm; 250 × 4 mm I.D.). Elution: gradient from 75:25 methanol–water to 100% methanol in 20 min. Detection: UV filter photometer at 254 nm.

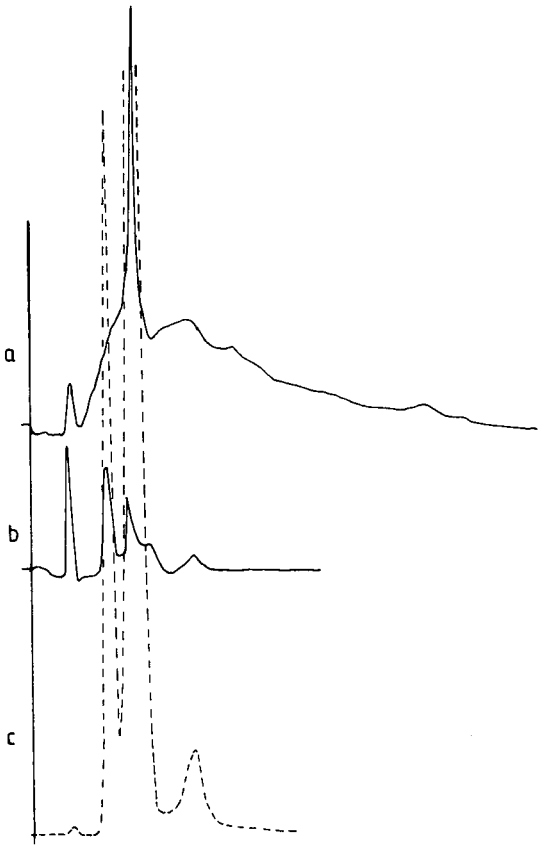

Fig. 6. HPLC profiles of the NHC IV fraction (a), its 3,5-dinitrobenzoates (b) and its 2,4-dinitrophenylhydrazones (c). Column: Vertex–LiChrosorb RP-18 (7 μm; 250 × 4 mm I.D.). Eluent: acetonitrile–water (60:40). Detection: UV filter photometer at 254 nm.

acetonitrile and water (60:40) or a gradient elution with methanol–water. The formation of 2,4-dinitrophenylhydrazones from C=O residues of the NHC III fraction is shown in Fig. 5. In Fig. 6 the formation of 2,4-dinitrophenylhydrazones from C=O residues and 3,5-dinitrobenzoates from OH residues in the NHC IV fraction is presented.

TABLE III

REACTION CONDITIONS AND YIELD OF HYDROCARBONS FROM TWO HYDROGENATION STEPS

	Step 1	Step 2
Amount of charge (g)	0.6	0.3
Reaction temperature (K)	653	698
Reaction time (min)	20	60
Catalyst	Fe^{II} sulphate	
Pressure of hydrogen at room temperature (MPa)	10	10
Yield of hydrocarbons (%)	32.1	32.4

Hydrogenation of the NHC

The NHC of sample 1 were carefully hydrogenated, taking special precautions to avoid side-effects of the mashing oil. The substances were first adsorbed on γ-aluminium oxide and were hydrogenated in two steps (Table III) in a 50-ml autoclave. The hydrocarbons formed in each step were separated by extrography, semipreparative and analytical HPLC (Figs. 7 and 8), and cGC. The structure-type composition found for each of the hydrogenation steps is listed in Tables IV and V.

It is evident that indanes, acenaphthenes, fluorenes and diphenyls were not formed in the second hydrogenation step. The unconverted NHC from both steps were characterized by ^{1}H-NMR (Fig. 9 and Table VI).

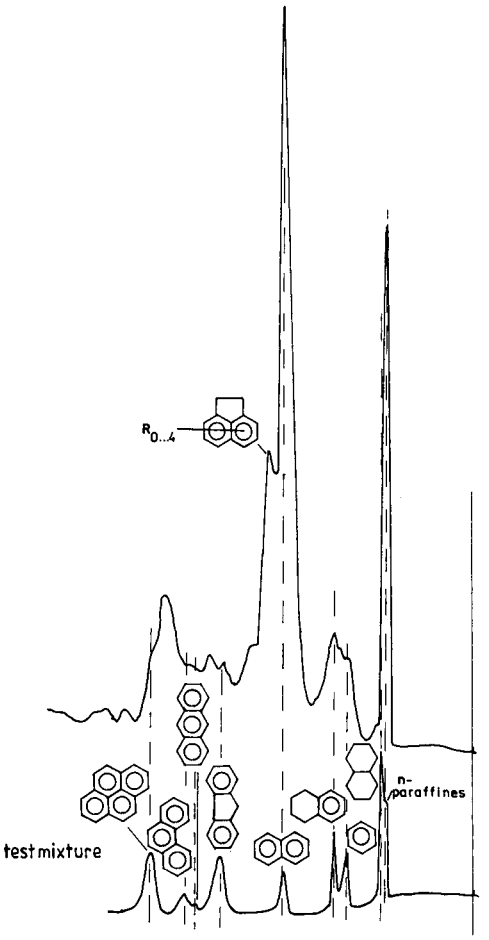

Fig. 7. Overview HPLC profile of the hydrocarbons of hydrogenation product 1 in comparison to a test mixture. For chromatographic conditions, see Fig. 2A.

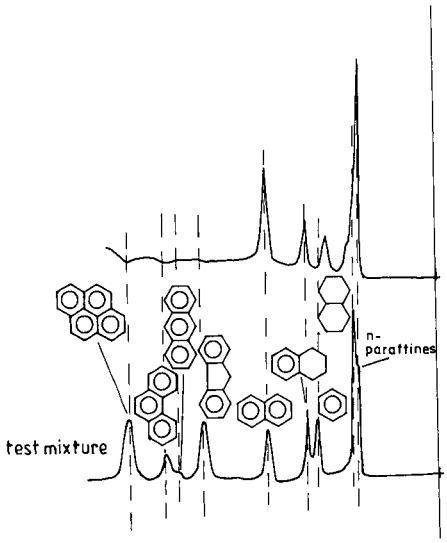

Fig. 8. Overview HPLC profile of the hydrocarbons of hydrogenation product 2 in comparison to a test mixture. For chromatographic conditions, see Fig. 2A.

TABLE IV

STRUCTURE-TYPE CHARACTERISTICS OF HYDROCARBONS IN THE PRODUCT OF THE HYDROGENATION STEP 1 (SEE ALSO FIG. 7)

Structure type	Content (%)	Qualitative composition
sCH	35.2	Paraffins > naphthenes
1 N-Ar	17.4	Alkylbenzenes, tetralenes/indanes
2 N-Ar	17.7	Naphthalenes, acenaphthenes, diphenyls, fluorenes
3 N+MN-Ar	24.4	Phenathrenes, pyrenes, chrysenes, polycyclic aromatics

TABLE V

STRUCTURE-TYPE CHARACTERISTICS OF HYDROCARBONS IN THE PRODUCT OF THE HYDROGENATION STEP 2 (SEE ALSO FIG. 8)

Structure type	Content (%)	Qualitative composition
sCH	28.6	Paraffins
1 N-Ar	18.7	Alkylbenzenes, tetralenes
2-N-Ar	27.1	Naphthalenes
3 N+MN-Ar	19.4	Polycyclic aromatics

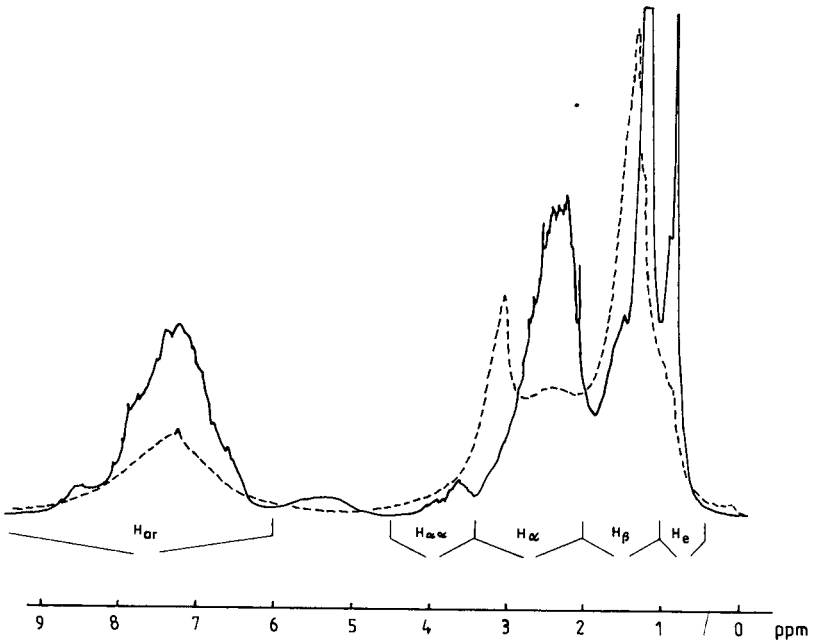

Fig. 9. ^{1}H-NMR spectra of NHC after (———) the first and (---) the second hydrogenation step.

TABLE VI

SPECTRAL CHARACTERISTICS OF NHC AFTER THE HYDROGENATION STEPS

Fraction	Hydrogen content (%)			
	H_{ar}	H_{α}	H_{β}	H_{e}
NHC before hydrogenation	9.44	22.04	51.12	17.40
NHC after step 1	26.05	28.73	33.42	10.13
NHC after step 2	14.17	34.90	50.97	–

CONCLUSIONS

Hydroxyl and carboxyl residues in NHC products of brown coal refining can be determined by derivatization and reversed-phase HPLC of the derivatives.

Circumstantial evidence for the existence of C–O–C residues can be deduced from the data of mass and FT-IR spectrometry.

The oxygen functional groups of NHC from brown coal refining products are bonded to aliphatic and naphthenic structures.

Heterocompounds containing aliphatic structures are preferentially hydrogenated to paraffins. Heterocompounds containing naphtheno-aromatic structures are hydrogenated by elimination of oxygen functional groups to aromatic and naphthenic hydrocarbons.

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CHROMSYMP. 2112

Isolation of chlorinated dibenzothiophenes by high-performance thin-layer chromatography

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ABSTRACT

Chlorinated dibenzothiophenes can cause environmental problems analogous to chlorinated dibenzodioxins and dibenzofurans. In the analysis of chlorinated dibenzodioxins by gas chromatography–mass spectrometry some unknown chlorinated compounds have been found. The chlorinated dibenzothiophenes have the same *m/z* values as the corresponding dioxins in low-resolution mass spectrometry and they are found in same planar aromatic compound fraction in alumina and carbon column chromatography. Some chlorinated dibenzothiophenes were synthesized to serve as model compounds in analytical work. High-performance thin-layer chromatography (TLC) with different plates and several eluents was tried to find a separation method for the chlorinated dibenzothiophenes. The experiments showed that reversed-phase TLC with RP-18 plates and acetonitrile–water eluents could separate the chlorinated dibenzothiophenes from many interfering compounds but not completely from complex environmental samples.

INTRODUCTION

Chlorinated aromatic compounds are present as harmful compounds in the environment. The chlorine derives mainly from anthropogenic activities and is incorporated in the planar aromatic compounds (PAC) structures during combustion processes. Chlorinated dibenzothiophenes can cause environmental problems analogous to chlorinated dibenzodioxins and dibenzofurans. In analysing chlorinated dibenzodioxins and dibenzofurans by gas chromatography–mass spectrometry (GC–MS) some unknown chlorinated compounds have been found which may be planar aromatic sulphur compounds. Chlorinated dibenzothiophenes in the purification and fractionation process that we use are found in the same fraction as the corresponding dioxins and dibenzofurans, hence these compounds may represent some of the unknown compounds in this fraction [1,2]. The chlorinated dibenzothiophenes have the same *m/z* values as the corresponding dioxins in high-resolution GC–low-resolution MS. A resolution of about 20 000 is needed to separate, for example, tetrachlorodibenzothiophenes (MW = 319.87880) from tetrachlorodioxins (MW = 319.89650).

The separation of the polyaromatic sulphur- and chlorine-containing compounds from interfering compounds in GC–MS and in GC, although a flame photometric detector in the sulphur mode was used, has been found to be difficult. Different liquid chromatographic fractionations have to be applied to obtain the

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required fraction with minimum amounts of interfering compounds [3].

In environmental samples, dibenzothiophenes usually occur in a complex mixture of organic compounds, so the first step is to separate the non-polar aromatic compound fraction which contains many kinds of alkylated and chlorinated compounds. This is usually done by silica gel or alumina column chromatography using hexane and hexane–dichloromethane as eluents. There is no unambigious method for separating the sulphur- and/or chlorine-containing compounds from other aromatic compounds in this fraction [3].

The complexation of organic sulphur compounds by means of copper, mercury, palladium and other metal salts has been used to separate the former. $PdCl_2$ is a good complexing agent for aromatic sulphur heterocycles, but further analysis of the palladium–sulphur compound complexes was found to be impractical owing to difficult degradation (the complex cannot be analysed by GC, GC–MS or high-performance liquid chromatography) [3–5].

In the mass spectrometric analysis of chlorinated and methylated aromatic sulphur compounds, the alumina or carbon column purified PAC fraction from environmental samples still contains many interfering compounds because an electron impact mass spectrometer is not sufficiently selective for compounds with a high electron affinity or sulphur to be able to confirm these compounds as chlorinated dibenzothiophenes. The concentrations of these compounds, if present in environmental samples, are very low compared with the other compounds in the same fraction, so better separation methods are needed.

EXPERIMENTAL

Compounds and samples

Several methylated and chlorinated dibenzothiophenes were synthesized for use as model compounds in the development of separation methods and in environmental and toxicological analysis of these compounds. Some of the lower isomers were obtained as pure compounds but mainly the synthetic products were mixtures of several different isomers, mainly with the same degree of chlorination or methylation.

The following compounds were prepared: 2-, 3- and 4-chlorobiphenyl, 2,4,5-trichlorobiphenyl, a mixture of 2,3,3′,4- and 3,3′,4,4′-tetrachlorobiphenyl, 2,4,5-trichlorodibenzothiophene and mixtures that contained several trichlorinated and tetrachlorynated biphenyls and dibenzothiophenes. The preparation of the model compounds is described elsewhere [2]. Pure dibenzothiophene was purchased from Fluka and dibenzothiophene 5,5-dioxide (sulphone) was prepared from this by oxidation.

Different non-polar aromatic compounds and planar aromatic compound fractions were obtained from environmental samples [1,6]. The non-polar aromatic compound fractions were originally prepared from environmental samples such as sediments, fish, mussel and oils for the analysis of polycyclic aromatic hydrocarbons and some non-polar aromatic oil residue compounds and the planar aromatic compound fractions from biological samples for the analysis of dioxins and dibenzofurans.

TABLE I

TLC PLATES AND ELUENTS USED

TLC plate	Eluents
Normal-phase	
Aluminiumoxid	Hexane, dichloromethane, light
Kieselgel	petroleum, toluene, acetone
NH_2	
Reversed-phase	
NH_2	Acetonitrile–water, methanol–water, ethanol–water
RP-18	(100:0, 90:10, 80:20 and 70:30)

Thin-layer chromatography

Chemicals were spotted with the aid of microsyringes on high-performance TLC plates. Chromatograms were obtained by ascending elution with mixtures of different solvents in closed tanks after equilibration with the aid of wet filter-paper. Aromatic UV-quenching substances were detected as dark or violet spots on fluorescent plates on illumination with UV radiation at 254 nm.

The following plates were used: Kieselgel 60 F_{254} (10 × 20 cm, thickness 0.25 mm, Merck, Art. 11846), Aluminiumoxid 60 F_{254} (20 × 20 cm, thickness 0.25 mm, Merck, Art. 5713), NH_2F_{254} für die Nano-DC (10 × 10 cm, thickness 0.2 mm. Merck, Art. 15647) and RP-18 $F_{254}S$ (5 × 20 cm, thickness 0.25 mm Merck, Art. 15683).

In normal-phase chromatography, with Aluminiumoxid, Kieselgel and NH_2 plates were used with several organic solvents and their mixtures. In reversed-phase chromatography, NH_2 and RP-18 plates were tried with different acetonitrile–water, ethanol–water and methanol–water mixtures (Table I).

RESULTS AND DISCUSSION

The NH_2 TLC plates were used in both the reversed- and normal-phase modes. In reversed-phase TLC different mixtures of methanol–water, acetonitrile–water and ethanol–water were tried. In normal-phase TLC organic solvents such as hexane, dichloromethane, light petroleum and toluene were used.

In RP-NH_2 TLC no separation could be achieved with methanol–water mixtures from methanol–water (1:1) to pure methanol. Oxidized sulphur compounds such as dibenzothiophene 5,5-dioxide were not eluted at all and other aromatic compounds, irrespective of the sulphur or chlorine content, had the same relative retention times, increasing nearly linearly with the increasing methanol concentration in the eluent. With ethanol–water eluents the relative retention times were higher but the separation of the components studied was not improved. Acetonitrile–water eluents could not be used to elute the NH_2 TLC plates as they did not ascend linearly on the plates.

In normal phase NH_2 TLC with hexane the chlorinated biphenyls and dibenzothiophenes were found to elute faster than the corresponding non-chlorinated compounds. However, the compounds could not be separated completely into these two groups. Dichloromethane eluted all the chlorinated and non-chlorinated com-

TABLE II

RP-18 TLC RESULTS (R_F VALUES) WITH ACETONITRILE–WATER ELUENTS

Acetonitrile–water	Sulphones	1–3-PAH	Cl-BP	Cl-DBT
100	0.64–0.68	0.57–0.62	0.43–0.49	0.48–0.54
90:10	0.64–0.69	0.52–0.58	0.35–0.40	0.42–0.49
80:20	0.55–0.64	0.38–0.48	0.18–0.26	0.28–0.35
70:30	0.46–0.54	0.27–0.34	0.11–0.16	0.21–0.26

pounds without any difference in retention times. Only oxidized compounds were more retained. Hexane–dichloromethane eluents did not improve the separation.

In reversed-phase TLC with RP-18 plates and acetonitrile–water eluents, the chlorinated dibenzothiophenes could be separated from chlorinated biphenyls, some oxidized dibenzothiophene derivatives and many other polycyclic aromatic compounds. Table II gives the results of RP-18 TLC experiments with acetonitrile–water for a mixture of chlorinated dibenzothiophenes (Cl-DBT), chlorinated bihenyls (Cl-BP), some low-molecular-weight aromatics without sulphur or chlorine (1–3-PAH) and some oxidized dibenzothiophenes (sulphones). With methanol–water eluents no separation could be achieved.

RP-18 TLC can be used to isolate the chlorinated dibenzothiophenes from the synthetic mixtures or from some relative simple environmental samples (some air or water samples), but not from fractions from complex polluted biological samples that contain hundreds of different aromatic compounds, many of which contain chlorine or sulphur. RP-18 TLC can be used apparently to decrease the number of compounds in these fractions. No separation of the examined compounds was achieved with alumina or silica plates.

CONCLUSIONS

Individual polycyclic aromatic hydrocarbons can be separated and analysed by RP-TLC using methanol–acetonitrile–water eluents [7–11].

Dibenzothiophenes as a separate group cannot be separated from the other compounds in the non-polar aromatic compound fraction using RP-TLC, but when oxidized to the corresponding sulphones to increase their polarity the separation can be achieved. Subsequent quantitative analysis must be done using high-performance liquid chromatography or GC–MS with a polar column [12]. However the sulphones are highly polar non-volatile and may be thermolabile, and hence cannot easily be analysed by GC.

The PAC fractions extracted from environmental samples are very complex and contain many different kinds of alkylated and chlorinated aromatic non-polar compounds. It is not possible to isolate chlorinated dibenzothiophenes as a separate group from fractions produced by extracting complex environmental samples using only TLC. However, by using TLC we can apparently decrease the number of different compounds in the fraction containing the chlorinated dibenzothiophenes.

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CHROMSYMP. 2304

Separation of minoxidil and its intermediates by overpressured layer chromatography using a stationary phase bonded with tricaprylmethylammonium chloride

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ABSTRACT

The retention behaviour of minoxidil (2,4-diamino-6-piperidinopyrimidine-3-oxide) and its intermediates (2,4-diamino-6-hydroxypirimidine, 2,4-diamino-6-chloropyrimidine and 2,4-diamino-3-N-oxo-6-chloropyrimidine) was studied by using silica gel layers impregnated with tricaprylmethylammonium chloride (TCMA). The retention of the compounds increases with increasing concentration of TCMA adsorbed on the silica gel. The pH and the ionic strength of the eluents do not affect the retention at all. The retention of the solutes decreases with increasing methanol content of the eluent, because of the TCMA-desorbing effect of methanol. On the basis of these and earlier findings, it was concluded that no ion-pairing occurs during the separation. A monolayer is formed on the silica surface at 0.1–0.2 *M* TCMA concentration in the impregnating solution, and hydrophobic interactions play an important role in the separation mechanism.

INTRODUCTION

The use of quaternary ammonium salts for the separation of different compounds in high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) is widespread. Tricaprylmethylammonium chloride (TCMA, Aliquat 336S), which has been applied as an active component of liquid membrane ion-selective electrodes, *e.g.*, salicylate-selective electrodes [1–4], proved very useful in the separation of different amino- and nitrosalicylic acids [5]. The chromatographic layers were impregnated with a methanolic solution of TCMA and the eluent did not contain any TCMA. Our further experience led us to study the retention characteristics of different basic compounds, *e.g.*, minoxidil (2,4-diamino-6-piperidinopyrimidine-3-oxide, Min) and its intermediates (2,4-diamino-6-hydroxypyrimidine, 6-OH; 2,4-diamino-6-chloropyrimidine, 6-Cl; and 2,4-diamino-3-N-oxo-6-chloropyrimidine, 3-N-oxo) (Fig. 1).

For development of the chromatograms, overpressured layer chromatography (OPLC), developed by Tyihák and co-workers [6–8], was used. Using OPLC one run takes only a few minutes, which is very important for in-process control, *e.g.*, in the production of minoxidil via its intermediates.

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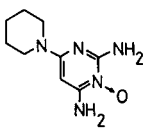

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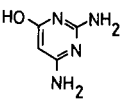

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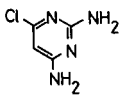

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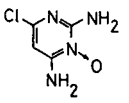

IV

Fig. 1. Structures of minoxidil (I) and its intermediates: 2,4-diamino-6-hydroxypyrimidine (II), 2,4-diamino-6-chloropyrimidine (III) and 2,4-diamino-3-N-oxo-6-chloropyrimidine (IV).

This work was concerned with the separation of the above-mentioned compounds by using silica gel layers impregnated with TCMA; in addition, we attempted to elucidate the mechanism of the separation mode.

EXPERIMENTAL

Apparatus

For development of the chromatograms, a Chrompres[25] overpressured layer chromatograph (Labor Instruments, Budapest, Hungary) was used, at a membrane pressure of 20 bar and a flow-rate of 1 ml min^{-1}. For TCMA adsorption studies, an OK 102/1 conductivity meter (Radelkis, Budapest, Hungary) was used. The spots were revealed under a UV lamp at 254 nm.

Chemicals and reagents

Silica gel plates were obtained from E. Merck (Darmstadt, Germany). Three sides of the plates were impregnated with Impres (Labor Instruments) for OPLC development and the layers were developed with methanolic TCMA overnight.

TCMA (Aliquat 336S) was purchased from Fluka (Buchs, Switzerland). Minoxidil and its intermediates were laboratory-made products of recrystallized quality; 1 μl of a 2 mg ml^{-1} methanolic solution was spotted. Methanol was of HPLC quality from E. Merck. All other chemicals were of analytical-reagent grade and were used as received.

RESULTS AND DISCUSSION

Retention on silica gel layers bonded with TCMA

The silica gel layers were impregnated with methanolic TCMA at concentrations from 0.005 to 0.2 *M* overnight. After evaporation of the methanol, the sample substances were spotted and the chromatograms were developed by OPLC as described under Experimental.

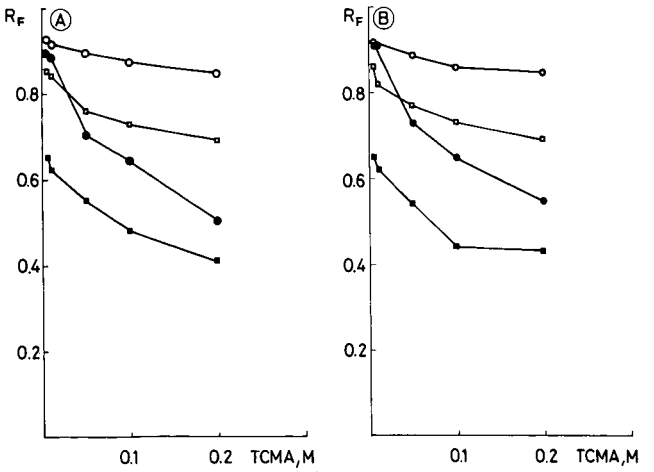

Fig. 2. Effect of TCMA concentration of impregnating solution on retention with methanol–aqueous Britton–Robinson buffer (50:50) eluent: (A) at pH 2.39; (B) at pH 9.40 (■) I; (○) II; (●) III; (□) IV (compound numbers as in Fig. 1).

Fig. 2 shows the effect of the TCMA concentration on the retention of minoxidil and its intermediates at pH 2.39 and 9.40. The eluent was aqueous Britton–Robinson buffer (0.05 *M*)–methanol (50:50). With increasing TCMA concentration, the retention increases for all the derivatives. Good separation can be achieved from 0.1 *M* TCMA. The efficiency of the separation (calculated in HETP values) is of the same order of magnitude for all the compounds as with 2.5% diethylamine on reversed-phase layers. It is notable that the HETP values are 2–3 times larger with 0.2 *M* than with 0.1 *M* TCMA. The pH of the eluent does not affect the retention.

Fig. 3A illustrates the effect of the methanol concentration on the retention.

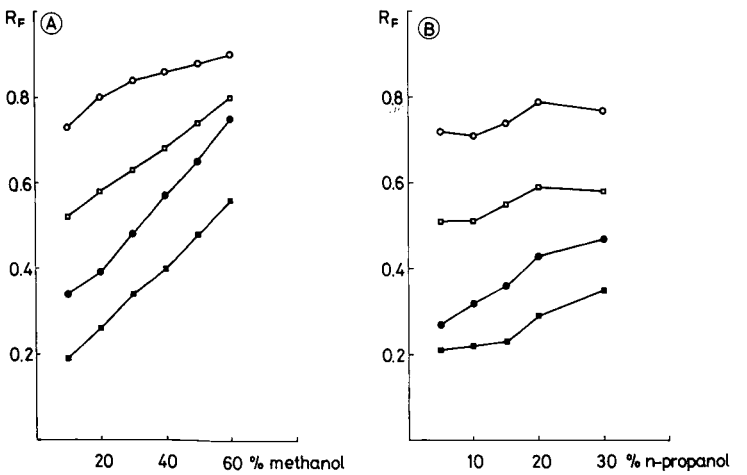

Fig. 3. Effects of (A) methanol and (B) *n*-propanol content of the eluent on retention on silica gel layers impregnated with 0.1 *M* TCMA. Symbols as in Fig. 2.

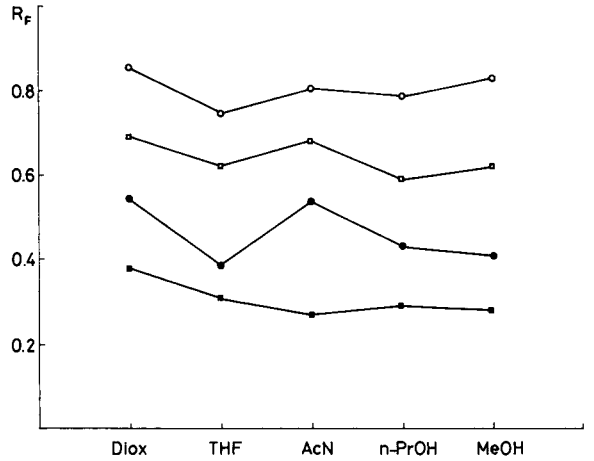

Fig. 4. Effects of different organic solvents on retention on silica gel layers impregnated with 0.1 *M* TCMA in eluents containing 80% water. Symbols as in Fig. 2. Diox = Dioxane; THF = tetrahydrofuran; ACN = acetonitrile; n-PrOH = *n*-propanol; MeOH = methanol.

The silica gel was impregnated with 0.1 *M* TCMA and the pH of the eluent was 2.39. From 10 to 60% methanol, the retention decreases almost linearly with increasing methanol content. With *n*-propanol instead of methanol, the retention of all the compounds decreases only slightly from 5 to 30% *n*-propanol content (Fig. 3B). At 30% and higher *n*-propanol concentrations, the shape of the spots becomes irregular. With other solvents instead of methanol, in a ratio of 8:2 with aqueous buffer solution at pH 2.39, the retention changes according to Fig. 4. No pronounced effect was observed in any solvent studied.

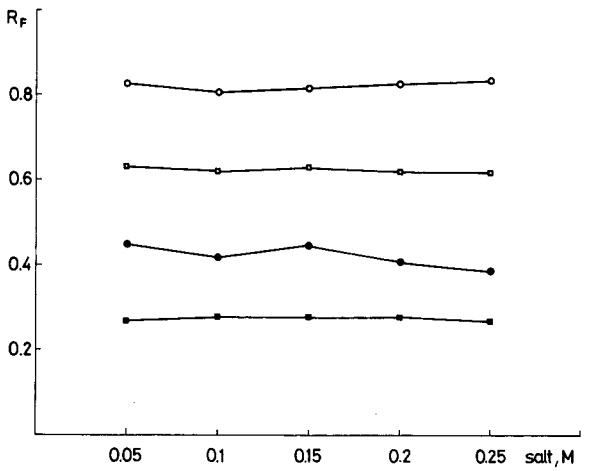

Fig. 5. Effects of ionic strength on retention on silica gel layers impregnated with 0.1 *M* TCMA in methanol–water (20:80) eluent. Symbols as in Fig. 2.

The ionic strength of the eluent, which was adjusted with Britton–Robinson buffer (0.05 *M* constant concentration) plus sodium chloride in various concentrations, does not affect the retention (Fig. 5).

For practical use, the best conditions are a silica gel layer impregnated with 0.1 *M* methanolic TCMA with eluents containing 10–50% of methanol and 90–50% of water. The R_s values are as follows with an eluent containing 10% of methanol: Min/6-OH = 7.94, Min/6-Cl = 2.50, Min/3-N-oxo = 4.83, 6-OH/6-Cl = 7.36, 6-OH/3-N-oxo = 3.50 and 6-Cl/3-N-oxo = 3.36; and with an eluent containing 50% of methanol: Min/6-OH = 5.67, Min/6-Cl = 2.77, Min/3-N-oxo = 3.76, 6-OH/6-Cl = 3.58, 6-OH/3-N-oxo = 2.11 and 6-Cl/3-N-oxo = 1.35. The spots are compact and the presence of TCMA does not affect the UV absorption characteristics of minoxidil and its intermediates.

On impregnation of the silica gel layers with 0.1 *M* cetrimide (cetyltrimethylammonium bromide) instead of 0.1 *M* TCMA, the R_F values of the compounds are similar, or slightly lower on the cetrimide-bonded layers with Min and the 6-OH derivative (Fig. 6). The compactness of the spots and the efficiency are the same with either TCMA or cetrimide.

Several experiments were performed on RP-18 reversed-phase layers. Using eluents containing 60–95% of methanol, Min can be separated from its intermediates, but the intermediates cannot be separated from each other. In acetonitrile–water eluents a 10–30% water content is suitable for the separation of all the compounds. The efficiency is greatest with a 30% water content. At lower water contents the spot diameters are large, indicating interactions between the solutes and the residual silanol groups. The presence of diethylamine or triethylamine in the eluent affects the efficidency only with Min, especially above 1.5%. The large elongated spots of the intermediates are not suitable for quantitative measurements.

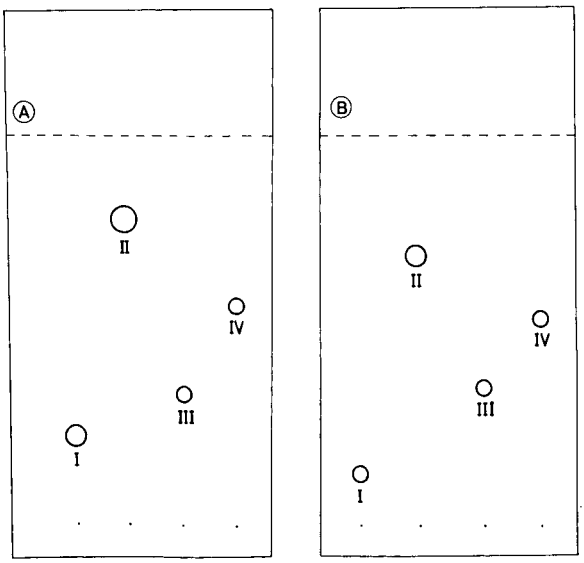

Fig. 6. Retention of minoxidil and its intermediates on silica gel layers impregnated with (A) 0.1 *M* TCMA and (B) 0.1 *M* cetrimide in methanol–water (20:80) eluent. Compound numbers as in Fig. 1.

Adsorption and desorption characteristics of TCMA on silica gel layers

Szepesi and co-workers [9–11] discussed in detail the adsorption behaviour of cetrimide on different layers. They concluded that the relative retention of different acidic compounds was highly dependent on the reagent concentration of the solution used for the immersion of the layers.

Tomkinson *et al.* [12] studied the retention behaviour of several dihydroxybenzoic acids on reversed-phase (C_2-bonded) HPTLC plates impregnated with tetra-*n*-butylammonium bromide (TBA), tetramethylammonium bromide (TMA) or cetrimide. Coating the plate with TBA or cetrimide gave the best chromatographic results, but these were largely ineffective in the eluent. TMA, in contrast, provided satisfactory results only when it was present in the eluent.

Our earlier investigations [5] with different amino- and nitrosalicylic acids on silica gel layers impregnated with TCMA gave the same results, *i.e.*, the retention was dependent on the TCMA concentration in the impregnating solution. In our case the eluent did not contain any TCMA. The pH of the eluent had no effect on the retention. Tomkinson *et al.* [12] also observed that the solvent pH is not an important factor in determining the final R_F of dihydroxybenzoic acids. This result was surprising in comparison with ion-pairing HPLC separation, where the effective concentrations of the ion-pairing reagent used are much lower and the pH plays a dominant role in the control of the retention.

On the basis of these findings, it seems that no ion pairing occurs during the separation. The separation mechanism is closely connected with the adsorption–desorption characteristics of the quaternary ammonium compound on the layer; further, there are interactions between the adsorbed quaternary ammonium compound and the solute.

To clarify this question, we determined the adsorbed TCMA concentrations on silica gel (and other) layers. For this purpose, a calibration graph was constructed on the basis of the conductivity of the methanolic TCMA solutions. The graph is linear from 0 to 0.01 *M* TCMA. The layers were impregnated with methanolic TCMA overnight. After drying, the silica gel was scraped off the plates along the horizontal axis in 1-cm strips. One strip was taken from near the start-line, another from the middle of the layer and the last from near the solvent front. The strips were then treated with 30–40 ml of methanol under reflux on a water-bath for 2 h. After cooling, the volume of the suspension in a volumetric flask was diluted to 50 ml with methanol and the conductivity was measured. Four TCMA concentrations (from 0.05 to 0.3 *M*) were used for the experiments. There was no difference between the conductivities of the strips taken from different sites of the layer. This means that no TCMA concentration gradient exists along the layer after the impregnation; a uniform bonding is formed on the layer.

The relationship between the concentration of the impregnating solution and the amount of TCMA adsorbed calculated in grams per gram of sorbent is illustrated in Fig. 7. The adsorbed TCMA concentration increases almost linearly at impregnating solution concentrations up to 0.1 *M*. Above 0.1 *M*, a slight increase can be observed. This is in accordance with the TCMA concentration (in the impregnating solution) *vs.* R_F values (Fig. 2). The quaternary ammonium compound is bonded to the silanol groups, and it is very likely that at a certain impregnating solution concentration a TCMA monolayer is formed on the silica gel surface. This concentration

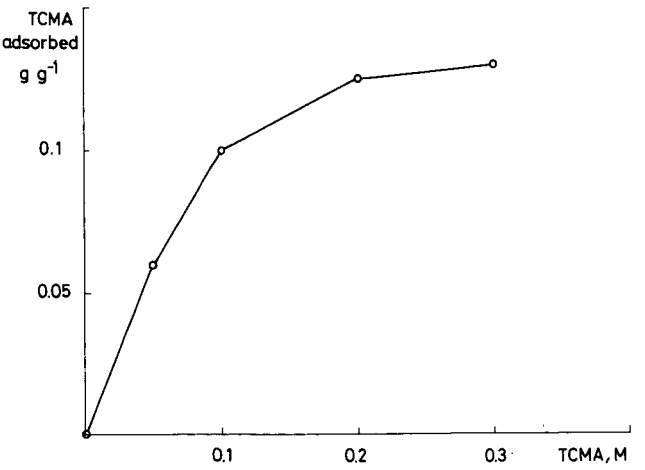

Fig. 7. Amount of TCMA adsorbed on silica gel layer (g g^{-1}) *vs.* TCMA concentration of impregnating solution.

seems to be between 0.1 and 0.2 *M*. As a consequence, the polarity of the surface decreases and the retention of the solutes increases. A hydrophobic interaction can be taken into account between the capryl groups and the solutes. This is why the pH of the eluent has no effect on the retention.

Another question to be clarified is the retention-decreasing effect of methanol or *n*-propanol (Fig. 3). When the TCMA impregnating layers are developed with methanol–water eluents of different compositions and the TCMA concentration is measured on the layers, a concentration gradient can be observed. At the start-line, strong TCMA desorption occurs and the amount of TCMA remaining on the layer is dependent on the eluent composition. As expected, the concentration of the retained TCMA is the lowest at methanol–water (75:25), and it is about twice as high if the eluent contains 25% of methanol. This explains the characteristic R_F *vs.* methanol content curves (Fig. 3).

Near the front-line, TCMA accumulates on the layer, which is why a linear relationship does not exist for the R_F *vs.* methanol concentration curves for the 6-OH derivative. Furthermore, band broadening can be observed at high methanol concentrations (60% or more). This means that a silanophilic interaction is preferred with increasing methanol content in the eluent. The desorption of the quaternary ammonium compound makes the silanol groups free. The same processes can be taken into account on layers impregnated with other quaternary ammonium salts in comparable concentrations. The retention difference observed between cetrimide and TCMA-impregnated layers with Min and the 6-OH derivative is due to the special individual interaction between the solute and the cetyltrimethyl (cetrimide) and tricaprylmethyl (TCMA) groups. For a given quaternary ammonium compound, this individual interaction depends on the polarity of the solute.

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Dependence of the silanophilic effect on the concentration of preadsorbed salts and on the chemical structure of peptides in reversed-phase thin-layer chromatography

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ABSTRACT

The retentions of eighteen peptides in reversed-phase thin-layer chromatography were determined with methanol as the organic mobile phase. Before impregnation inorganic salts (LiCl, NaCl, KCl, $CaCl_2$ and $MgCl_2$) were preadsorbed on the silica support in the concentration range of 0.5–2 ml of 1 *M* salt solution per gram of silica. The majority of peptides showed a silanophilic effect: their R_M values first decreased to a minimum and then increased with increasing organic phase concentration. The retention of peptides depended quadratically on the methanol concentration and linearly on the salt concentration, on the hydrated ion radii and on the charge of the cation, that is, salts could not mask the silanophilic effect of free silanol groups even at higher concentrations. Principal component analysis (PCA) proved that the linear and quadratic forms of methanol concentrations have a similar effect on the retention of peptides; however, the salt concentration, the hydrated ion radii and charge of cations modify the retention differently. The PC variables did not correlate with the lipophilicity of peptides, proving the lipophilicity modifying effect of salts. No correlation was found between the parameters of quadratic functions fitted for salt-free and salt-containing systems, proving again the lipophilicity-modifying effect of salts. On the two-dimensional non-linear map of PC variables the peptides did not form cluster according to their structural characteristics, proving the participation of each peptide substructure in the peptide–salt interaction. However, the overall polarity of peptides influenced their salt sensitivity.

INTRODUCTION

In drug design, computer-assisted multivariate mathematical methods have gained growing importance and acceptance [1]. The lipophilicity of bioactive molecules is one of the physico-chemical parameters most frequently used in quantitative structure–activity relationship (QSAR) studies [2]. Reversed-phase thin-layer chromatographic (RP-TLC) methods have been extensively applied to determine the lipophilicity of many bioactive compounds [3]. To increase the accuracy of the lipophilicity determination, linear correlations have been calculated between the R_M values (characterizing the lipophilicity in RP-TLC) and the concentration of organic mobile phase in the eluent, the R_M value extrapolated to zero organic phase concentration (R_{M0}) was regarded as the most accurate estimate of the lipophilicity [4,5]. However, with peptides [6], quaternary amino steroids [7] and crown ether

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derivatives [8,9], no linear correlation was found between the R_M value and the concentration of the organic mobile phase. The R_M value decreased with increasing organic phase concentration in the lower concentration range, reached a minimum, and then increased with further increase of the organic phase ratio. This phenomenon was tentatively explained in terms of silanophilic effect: at higher organic phase concentrations, the solute molecules have an enhanced probability of access to the silanol groups uncovered by the impregnating agent. The interaction with the free silanol groups results in increased retention and an increased apparent lipophilicity [6]. It was assumed and in some instances proved experimentally that the adsorptive side-effect of free silanol groups can be eliminated or reduced by the addition of alkylamines [10] or salts [11] to the eluent. To our knowledge, the concentration of salts needed to eliminate the silanophilic effect and the impact of the various physico-chemical parameters of salts (ion charge and hydrated ion radii) on the elimination process have never been studied in detail. Reversed-phase chromatography has been extensively applied to separate peptides on both the analytical [12] and preparative [13,14] scale. The retention depended on the type [15] and density of the hydrophobic ligand [16]. Moreover, reversed-phase chromatography has been applied to the study of peptide behaviour at hydrophobic liquid–solid interface which mimic biological lipid bilayers. It helped to identify and characterize both the hydrophobic interaction sites and the existence of conformational equilibria of peptides such as β-endomorphin [17,18], luteinizing hormone-releasing hormone [19], myosin kinase analogues [20] and human growth hormone-related peptides [21,22].

The objectives of this work were to determine the effect of salts preadsorbed on a silica surface on the silanophilic effect and to elucidate the role of the salt concentration and that of ion charge and hydrated ion radii in the decrease in the silanophilic effect. We assumed that, owing to their amphipathic character, peptides are ideal test solutes to study the above effects.

EXPERIMENTAL

Kieselgel 60 H (Merck, Darmstadt, Germany) was used as a support. The salt solutions (0.5, 1 and 2 ml of 1 *M* NaCl, 1 ml of 1 *M* LiCl, KCl, $MgCl_2$ and $CaCl_2$ per gram of silica) were added to the silica before preparing the plates. Layers 0.25 mm thick were prepared on 20 × 20 cm glass plates and were dried overnight at room temperature and impregnated by predevelopment in 5% paraffin oil in *n*-hexane [23]. Untreated Kieselgel 60 H plates served as a control. We are well aware that the application of hand-made plates increases the inherent low reproducibility of TLC. However, the application of this method was motivated by the fact that the addition of salts cannot be carried out with acceptable accuracy under other experimental conditions.

The structures of the peptides are given in Table I. The peptides were dissolved in water–1-propanol (2:1, v/v) at a concentration of 2 mg/ml, and 2 μl of each solution were spotted on the plates. Methanol in water was applied as the organic mobile phase in the concentration range of 0–90 vol.% at 10% intervals. After development, the peptides were detected with ninhydrin. For each experiment, five parallel determinations were carried out.

For a given RP-TLC system, whenever the peptide remained at the start or was

TABLE I

STRUCTURES OF PEPTIDES

All amino acids had the L configuration. β-Abu = β-Aminobutyric acid; γ-Abu = γ-aminobutyric acid; γ-Ape = γ-amino-δ-methylhexanoic acid.

Compound No.	Structure	Compound No.	Structure
1	β-Abu–Ala	11	Glu–Cys–Gly
2	Gly–Gly	12	Thr–Ile–Pro
3	Phe–Ala	13	Pro–Thr–Ile–Pro
4	Ala–Ala	14	Trp–Ser–Tyr–Gly
5	γ-Ape–γ-Abu	15	Trp–Ala–Ile
6	γ-Abu–γ-Abu	16	Ala–Lys–Pro–Lys
7	Ala–β-Abu	17	γ-Glu–Cys–Gly
8	Ala–Thr	18	γ-Glu γ-Glu | | Cys–S–S–Cys | | Gly Gly
9	Gly–Leu–Gly		
10	Gly–β-Abu–Gly		

very near to the front (deformed spot shape), or the relative standard deviation of the five parallel determinations was higher than 10%, the data were omitted from the calculations. As our data indicated that the retention of peptides simultaneously depended on the methanol concentration in the eluent and on the concentration and on the type of the preadsorbed salt, stepwise regression analysis [24] was applied to select the variables that significantly influenced the retention.

The R_M value [25] of peptides defined by the following equation was taken as the dependent variable:

$$R_M = \log(1/R_F - 1) \tag{1}$$

The linear and quadratic forms of the methanol concentration, the amount of preadsorbed salt, the charge and the hydrated ion radii of the cation were taken as independent variables (total five independent variables). The acceptance limit for the selected independent variables was set at 95% significance. The inclusion of the quadratic form of the methanol concentration was motivated by the finding that the irregular retention behaviour of peptides can be well described by a quadratic correlation [26]. Stepwise regression analysis was carried out separately for each peptide. To evaluate the retention-modifying effect of salts, linear correlations were calculated between the slope values of the independent variables concerning the effect of methanol (linear and quadratic form of methanol concentrations) determined in salt-free and salt-containing systems.

To elucidate the similarities and dissimilarities between the retention behaviour of peptides and the chromatographic parameters, principal component analysis (PCA) was applied [27]. The application of various multilinear regression methods requires the presence or selection of a dependent variable. However, in many instances one is not interested in the dependence of one parameter (dependent variable) on the other parameters (independent variables), the aim rather being to find the relationship

between all parameters without one being dependent variable. PCA complies with these requirements. The advantages of its application are the clustering of the variables according to their relationship, the possibility of extracting one or more background variables which may have a concrete physico-chemical meaning and the decrease in the number of variables.

The peptides were taken as observations, and the chromatographic parameters (the five slope values of the independent variables of the stepwise regression analysis) served as variables. The application of each slope value was motivated by the fact that these values are indicators of the retention behaviour of peptides. To avoid the information loss caused by the normalization, PCA was carried out on the covariance matrix instead of the correlation matrix. When the stepwise regression analysis proved that the slope value of a given independent variable did not deviate significantly from zero, the zero value was included in the PCA. The two-dimensional non-linear map of PC loadings and variables was also calculated [28]. The peptides (or chromatographic parameters) showing similar retention behaviour form clusters on the two-dimensional map. In most instances the coordinates of the map (F_1 and F_2) do not have any concrete physical or physico-chemical meaning. They only show the distribution of the variables in a plane which was reduced from the multi-dimensional PCA space.

To assess the role of the lipophilicity of peptides in the retention, linear correlations were calculated between the first three principal component variables and the lipophilicity of peptides. The lipophilicity values of peptides were taken from ref. 29 or calculated accordingly.

RESULTS AND DISCUSSION

Peptides exhibited typical silanophilic retention behaviour, the R_M value decreasing with increasing methanol concentration in the lower concentration range, and then increasing with further increase in the methanol ratio (Fig. 1). Apart from the silanophilic effect, this biphasic retention behaviour may be due to the adsorption of methanol at higher concentrations on the modified silica, which then results in a normal phase-like surface. The effect of salts depended on their concentration; however, the salt could not eliminate the silanophilic effect even at fairly high concentration (Fig. 2), which indicates that parameters other than the presence of free (uncovered) silanol groups may have some impact on the irregular retention behaviour of peptides. The cation type (charge and hydrated ion radii) also influenced the silanophilic effect (Fig. 3), as will be explained later.

The results of stepwise regression analysis are given in Table II. The F values show that the equations selected fit the experimental data well, the significance level being over 99.9% in each instance. The calculations entirely support our previous qualitative conclusions. The methanol concentration (linearly and/or quadratically) significantly influenced the retention of each peptide, proving again the irregular retention behaviour of peptides and the existence of the silanophilic effect. However, the character of the correlation showed high variance. The significant differences between the slope values of peptides indicates that their retentions deviate significantly from each other, that is, the structural parameters of a peptide considerably influence the nature of the silanophilic effect. The concentration of preadsorbed salts, the ion

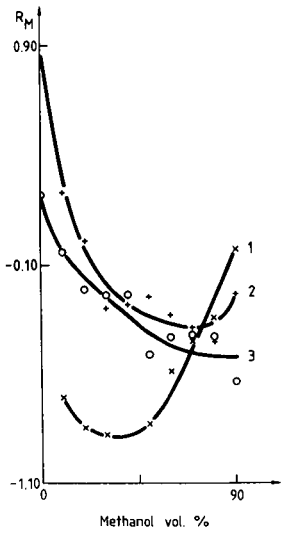
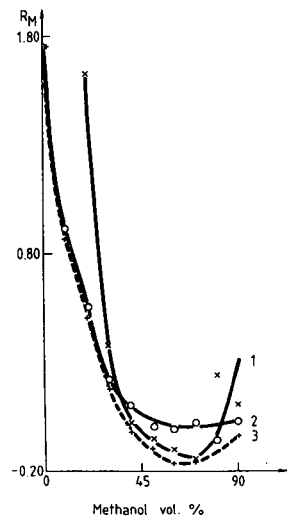


Fig. 1. Dependence of R_M value of some peptides on the methanol concentration in the eluent (1 ml of 1 *M* KCl per gram of silica). 1 = Peptide 2; 2 = peptide 12; 3 = peptide 3. For structures, see Table I.



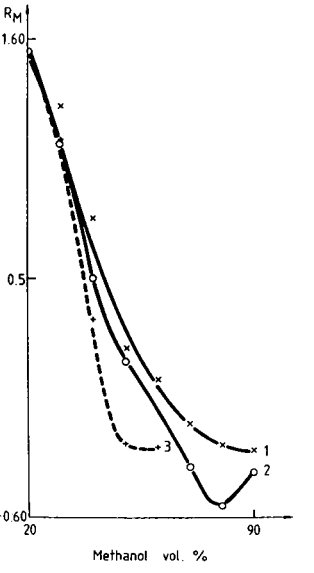


Fig. 2. Dependence of R_M value of peptide 14 on the methanol concentration in the eluent and on the amount of preabsorbed salt. Volume of 1 *M* NaCl per gram of silica: 1 = 2 ml; 2 = 1 ml; 3 = 0.5 ml.

Fig. 3. Dependence of R_M value of peptide 15 on the methanol concentration in the eluent and on the type of preadsorbed salt (1 ml of 1 *M* salt solution per gram of silica). 1 = $CaCl_2$; 2 = KCl; 3 = LiCl.

TABLE II

DEPENDENCE OF THE R_M VALUES OF PEPTIDES ON THE METHANOL CONCENTRATION (C) IN THE ELUENT AND ON THE PHYSICO-CHEMICAL CHARACTERISTICS OF SALTS

Results of stepwise regression analysis. $R_M = a + b_1C + b_2SC + b_3IR + b_4IC + b_5C^2$. SC = salt concentration; IR = hydrated cation radii; IC = cation charge.

Parameter[a]	No. of peptide[b]					
	1	2	3	4	5	6
n	42	47	47	52	67	65
a	−0.67	−1.13	−0.12	−0.78	0.15	0.03
$b_1 \times 10^{-3}$	n.s.[c]	n.s.	−24.2	5.30	−8.81	−7.14
$s_{b1} \times 10^{-3}$			2.78	6.85	1.67	1.79
p.c.			49.67	41.52	40.26	32.07
b_2	n.s.	0.13	n.s.	0.11	n.s.	n.s.
s_{b2}		0.06		0.05		
p.c.		12.20		12.25		
b_3	n.s.	n.s.	n.s.	−0.31	−0.24	−0.29
s_{b3}				0.10	0.08	0.08
p.c.				15.50	6.58	7.84
b_4	0.27	0.32	0.24	0.27	$8.26 \cdot 10^{-2}$	0.12
s_{b4}	0.07	0.06	0.05	0.05	0.03	0.03
p.c.	59.37	31.35	7.48	30.73	5.91	8.34
$b_5 \times 10^{-5}$	3.13	7.36	22.6	n.s.	11.0	12.4
$s_{b5} \times 10^{-5}$	1.21	0.78	3.00		1.78	1.93
p.c.	40.63	56.45	42.85		47.24	51.71
F	13.34	50.77	37.37	33.93	16.71	33.53
r^2	0.7388	0.7759	0.7181	0.7388	0.5148	0.6874
	7	8	9	10	11	12
n	63	52	50	50	27	47
a	−0.18	−0.44	−0.18	−0.67	−1.07	0.37
$b_1 \times 10^{-3}$	−4.74	−8.33	−17.9	−9.71	−11.4	−39.0
$s_{b1} \times 10^{-3}$	1.66	2.57	2.15	1.89	5.26	3.29
p.c.	26.94	12.59	47.58	34.33	28.46	52.29
b_2	n.s.	n.s.	n.s.	n.s.	0.26	n.s.
s_{b2}					0.10	
p.c.					9.73	
b_3	−0.22	n.s.	n.s.	n.s.	n.s.	n.s.
s_{b3}	0.07					
p.c.	7.39					
b_4	0.17	0.17	0.22	0.41	0.44	0.21
s_{b4}	0.03	0.05	0.04	0.04	0.09	0.06
p.c.	14.32	12.59	9.06	20.69	19.34	4.24
$b_5 \times 10^{-5}$	9.51	11.1	17.6	13.9	16.9	35.1
$s_{b5} \times 10^{-5}$	1.74	2.76	2.32	2.04	5.22	3.57
p.c.	51.34	48.30	43.37	44.97	42.47	43.47
F	41.29	12.91	30.18	68.60	16.82	62.04
r^2	0.7368	0.4414	0.6583	0.8141	0.7453	0.8088

TABLE II (*continued*)

Parameter	No. of peptide[b]					
	13	14	15	16	17	18
n	38	66	38	63	25	15
a	1.95	1.20	3.22	2.43	−1.19	−0.78
$b_1 \times 10^{-3}$	−25.7	−49.2	−111	−25.5	−47.4	n.s.
$s_{b1} \times 10^{-3}$	2.56	2.98	7.80	5.07	9.40	
p.c.	60.43	55.07	53.92	50.37	47.37	
b_2	−0.50	0.13	0.23	−0.29	1.39	n.s.
s_{b2}	0.13	0.06	0.07	0.10	0.65	
p.c.	22.55	2.07	2.15	7.87	6.14	
b_3	n.s.	n.s.	n.s.	−0.80	n.s.	n.s.
s_{b3}				0.22		
p.c.				9.75		
b_4	0.36	0.10	0.22	n.s.	0.69	n.s.
s_{b4}	0.13	0.05	0.06		0.14	
p.c.	17.02	1.84	2.20		15.62	
$b_5 \times 10^{-5}$	n.s.	38.7	74.3	17.1	35.9	14.2
$s_{b5} \times 10^{-5}$		3.13	6.69	5.32	10.78	
p.c.		41.01	41.73	32.03	30.87	
F	36.13	114.66	132.31	23.51	33.07	63.84
r^2	0.7559	0.8809	0.9396	0.6145	0.8630	0.8205

[a] Symbols: n = Number of independent observations (sample number); a = intercept (equal to the value of the dependent variable at zero concentration of the independent variables); b_{1-5} = slope values (change in dependent variable caused by a unit change in the independent variable); s_{b1-5} = standard deviations of the corresponding slope values; p.c. = path coefficient (%) = normalized slope values indicating the relative impact of the individual independent variables on the dependent variable independently of their dimension; F = calculated value of the F-test; r^2 = coefficient of determination indicating the ratio of variance explained by the independent variables.

[b] See Table I.

[c] Not significant.

charge and the hydrated ion radii of the cation sometimes, but not always, significantly influenced the retention. However, the path coefficient values (indicators of the impact of the given variable on the dependent variable independently of its dimensions) clearly showed that in most instances the effect of methanol concentration is much higher than that of salts.

The ions (we assume that the salts are more or less dissociated in water–methanol mixtures) occupying the free silanol groups on the silica surface modify the adsorptive capacity of the silica support. This effect prevails even after impregnation which proves indirectly that the paraffin oil does not cover all adsorption sites on the silica surface. We assume that the retention-modifying effect of salts is the result of at least two phenomena. The ions adsorb on the free silanol groups resulting in decreased retention. After saturating the available silanol groups, the non-adsorbed ions interact with the peptides, modifying their distribution between the stationary and the mobile phase (salting-out or salting-in effect). We assume that the ions with higher hydrated ion radii have a lower access to the active sites of the silica surface (smaller retention-decreasing effect), whereas a higher ion charge may result in a better adsorption capacity (enhanced retention-decreasing effect).

The observed retention behaviour is an interplay of the various interactions outlined above the exact molecular base of which is not well known and needs more investigation. These considerations explain why no linear correlation was found between the retention-modifying capacity of methanol in salt-free and salt-containing systems.

The first and second principal component explained 69.7 and 19.4% of the total variance, respectively, in other words there is a background variable (the first PC) which accounts for 69.7% of the effect of the five variables. Of course, the existence of a principal component does not necessarily mean the existence of a corresponding physico-chemical parameter but only indicates the mathematical possibility of such a parameter.

The two-dimensional non-linear map of PC loadings shows the similarities and dissibilarities of the five variables taking into consideration simultaneously the retention behaviour of all peptides (Fig. 4). The linear and quadratic forms of methanol concentration (points 1 and 5) are very close to each other on the map, indicating the similarity of their action on the retention.

The salt concentration, the ion charge and hydrated ion radii are widely separated on the map, suggesting that each of them separately influences the retention of peptides, that is, each variable is suitable for selectively modifying the retention.

The two-dimensional non-linear map of PC variables shows the clustering of peptides, taking into consideration simultaneously the effect of all chromatographic parameters (Fig. 5). The peptides did not form clusters according to the length of peptide chain or the presence of a bulky side-chain structure. This finding indicates that these molecular characteristics do not influence the salt sensitivity of peptides. The more polar peptides (alkaline peptide 16 and acidic peptide 17) are widely separated from each other and from the bulk of other peptides; however, the acidic peptide 18 does not show any deviating retention behaviour. This finding indicates that the salts

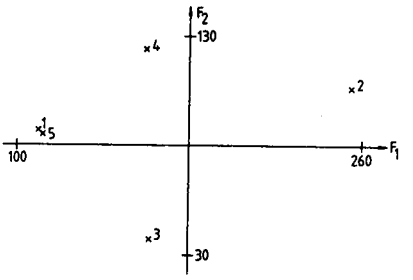


Fig. 4. Two-dimensional non-linear map of PC loadings. No. of iterations, 314; maximum error, $8.76 \cdot 10^{-3}$. 1 = Methanol concentration; 2 = salt concentration; 3 = hydrated cation radii; 4 = cation charge; 5 = square of methanol concentration.

Fig. 5. Two-dimensional non-linear map of PC variables. No. of iterations, 126; maximum error, $1.39 \cdot 10^{-2}$. Numbers refer to peptides in Table I.

have a greater effect on the retention of more polar peptides. This conclusion was supported by the fact that no significant linear correlation was found between the principal components and the lipophilicity of peptides.

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Quantitative two-dimensional thin-layer chromatography

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ABSTRACT

Two-dimensional thin-layer chromatography (TLC) is seldom used as a quantitative method in spite of its enormous potential, the main reasons being a single sample, a complicated solvent system optimizing procedure, and lack of adequative evaluation procedures. A special set of computer programs which enable scanning and evaluation TLC scanner is described. A whole TLC plate is scanned in linear mode with normal slit shape, for instance 0.2 × 8 mm, with as small as 0.5 to 1.0 mm steps between scans. Each run is processed as for a normal densitogram, the baseline is constructed, substracted, and the eventual peaks are located and integrated. In the next step a special program selects the correct positions of peaks, according to the biggest peak height, of all the scans. The selected scans are reintegrated and peak heights and areas are reported. Manual mode results were prepared in addition to the automatic mode. The operator selects the most convenient scan and integrates it using the cursors and chromatograms on the screen. The practical application of the scanning and evaluation programs is presented with quantitative determination of some phenolic components in propolis. Plates are developed with the solvent system: toluene–ethyl acetate–formic acid (75:15:3) and in the perpendicular direction with the solvent system: chloroform–methanol–formic acid (75:5:4). Identification and quantification of some characteristic flavonoids is performed with the 2-aminoethyldiphenylborinate/polyethylene glycol 4000 derivation reagent and external standards.

INTRODUCTION

Modern analytical chemistry strongly supports the development of procedures which yield the greatest output of information in the shortest possible time. One of these methods can also be an uncomplicated, multidimensional thin-layer chromatography (TLC) separation. Without the use of sophisticated instruments it is possible to separate complicated mixtures using different mobile phases in different directions on normal TLC plate. The real problem arises when the quantitative evaluation of two-dimensional chromatograms has to be performed [1–4]. TLC and two-dimensional TLC are commonly used in our laboratory, therefore we prepared a group of programs under the name "td/QTLC-pack".

This software pack consists of programs for scanning, automatic or manual integration, documentation and post-run calculation. The program is prepared for a computer-controlled scanner, CAMAG TLC Scanner II equipped with an AD converter and RS232 serial interface. The construction of the "td/QTLC-pack" is shown in Fig. 1. The densitometer is connected to a personal computer: IBM PC/XT or AT, with 640 kb RAM, serial interface, and CGA, EGA or Hercules graphics.

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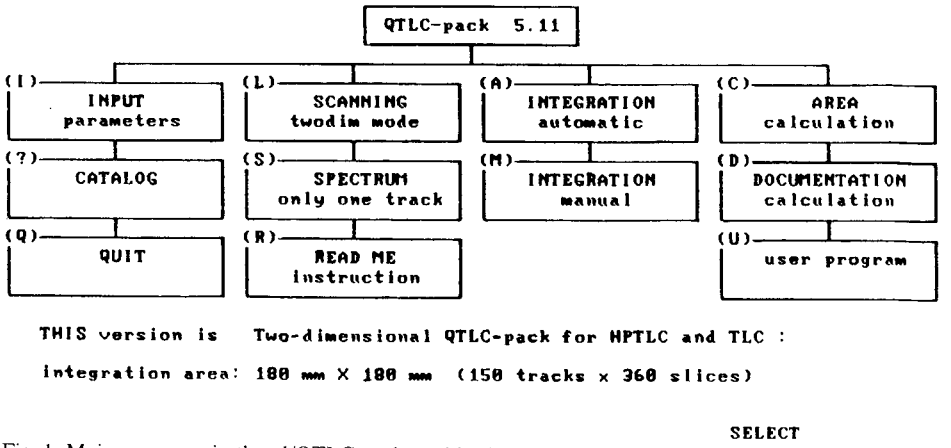

Fig. 1. Main programs in the td/QTLC-pack used in this work.

It is clear that only an image processing system which is able to collect at least 512 × 512 data points (pixels) from a plate is the correct answer to multidimensional quantitative TLC. As such systems are not commercially available, the only possible solution is data acquistion with a TLC densitometer.

Our routine work has revealed that scanning with a small square slit (0.5 × 0.5 mm), which is moved over the whole plate, is not the best solution. Scanning produces a large amount of noisy data which must be treated with a special smoothing program prior to being integrated. Since data acquisition is a long procedure (45 min), it is practically impossible to improve the signal-to-noise ratio with multiple scans. Thus small pixels can be used only with a video cameras.

We decided to try a different solution which yielded very good results in a relatively short time. The basic idea of our scanning is that a plate is scanned normally, as in linear TLC, with a slit the dimension of which is determined from the width of the spot, the width being slightly bigger than the measured spots [5,6]. Scans are repeated with small steps in the x direction, much smaller than the slit itself: for instance, the slit width is 5 mm and steps between scans are 0.5 mm (Fig. 2). In this mode the collected signal from the plate is already integrated in the x direction (slit length) during the scan in an analogous mode with improved signal-to-noise ratio. After data acquisition, the intergration program takes each lane, finds the cardinal points of the possible peaks present, constructs the baseline, substracts it from the signal and integrates areas under the curve, as with normal one-dimensional TLC. The correct position of each peak on the two-dimensional plate is located according to the greatest peak height. The integration program records peaks heights of each lane and compares them with the corresponding values from other lanes. The position of the biggest peak height is taken as the correct peak position in the y direction, and a corresponding raw data set is used for quantitative evaluation of this peak. This quantitative two-dimensional scanning mode gives good signal-to-noise ratio because the first part of integration is performed during the scan and only the second part of data processing is done by the computer in post-run data processing.

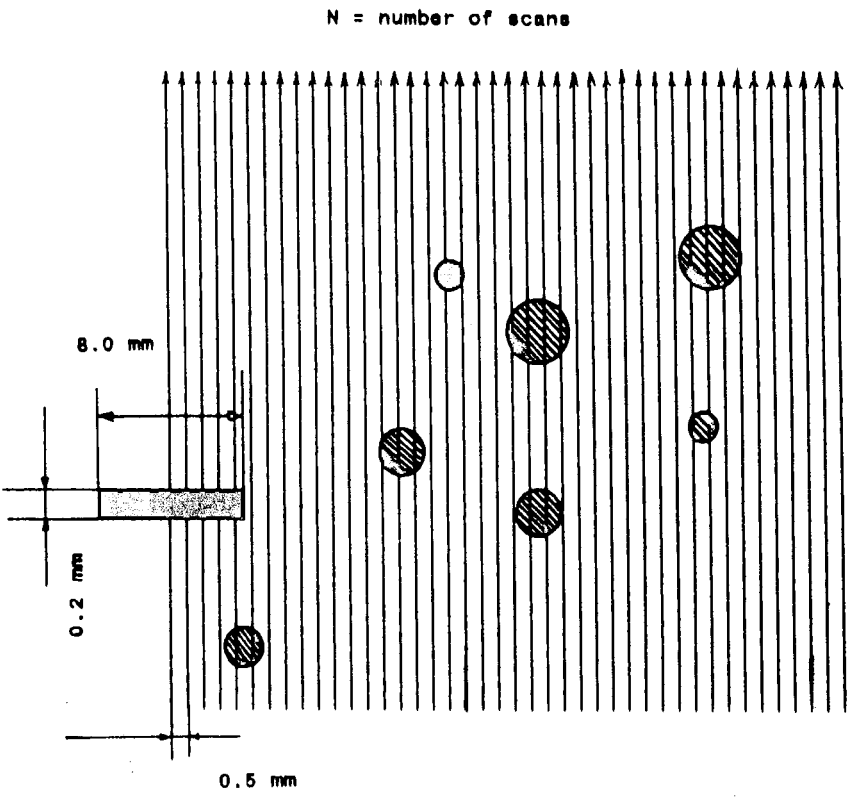

Fig. 2. The scanning principle of two-dimensional TLC, using a normal linear scanning mode.

td/QTLC-pack

Our interest in computer-controlled data acquisition and processing in TLC started nearly 20 years ago. Since then our own software system for computer controlled densitometry, the "QTLC-pack" has been developed. It was used on many different computers: HP9380, PDP 8, Apple II and IBM PC and also on different types of TLC separations: linear, circular, anticircular.

The td/QTLC software system was developed from this basic programing concept. It consists of four groups of programs. In the first group, there is an INPUT program. It is used for definition of all necessary parameters for scanning, integration, and calculation which are under the direct control of the operator. The programs for data acquisition are in the second group. The TDSCAN program collects raw data: 80–160 scans are taken from one plate, each scan being stored under the name RSCAN.X (x from 1 to 160). After collection, raw data are treated with the REDUCE program which reduces the number of measurements. In each scan the number of raw data points is reduced by a factor (N). The reduction results in less than 360 slices in one scan, regardless of the original number of data points. Each slice is the mean value of N original points. A new data set with 300 points is obtained from a chromatogram with scan length 90 mm and 900 measurements (0.1 mm steps).

Reduced data are stored on a disk under the name RSAMPLE.*Y*, where *Y* is a plate number.

A two-step scanning procedure has been selected in order to optimalize the use of a disk space (340 Kbyte disk on PC-XT). As separation on the TLC plate is not very large it is sufficient to have 360 points to succesfully handle all the separated peaks in a scan. Reduction helps in elimentation of the noise problem, where slices are used instead of points. The same integration algorithm can be used for data processing of all types of plates in TLC (150 × 150 mm) and high-performance (HPTLC) (70 × 70 mm), as there is always the same number of slices in a scan. In addition it helps to speed up the data processing. The PC, especially the XT version, is capable of performing all the calculations, but is slow when a lot of data are involved.

In the third group there are programs for automatic and manual intergration. The automatic integration program finds positions and relative intensity of peaks as a function of peak height. In a scan a baseline is constructed and subtracted before peak positions are set. After automatic mode determination, an operator can set additional, non-integrated peaks, or eliminates those already found.

The program for manual integration is prepared for situations where it is necessary to analyse only few spots among many substances on a plate. It is possible to set a baseline, peak height and area for each individual spot on the plate.

Post-run calculation is done in the fourth group. The TDAREA program reintegrates lanes with the selected spots. The TDDOC program documents the spots already integrated on a screen and printer. A short description of the practical operation of the td/QTLC-pack is given. With this we want to stress the importance of individual programming in the analytical laboratory. We hope that other analysts will start describing their approach to computer control of individual steps in chromatography. It is strange that most useful papers about digital data acquisition and integration are from a peroid when lab computers were rare. It seems that any interest in further implementation of computer power to analytical methods is nowadays the concern of producers of the instrument only – this is unfortunate as the developement of new, original computer-supported analytical procedures is possible only with the analysts who have computer knowledge and are capable of developing their own programs.

Our group is one of few groups still using their own programs for data processing in chromatography. These programs are not on the same professional level as some commercially available ones, but are very flexible and have original algorithms regarding laboratory manipulation. The software is started with the QTLC program. The computer prepares a shell for a pack and calls up the START program which connects all other programs (every other program is selected and started by it).

The INPUT program enters and corrects all the parameters used in QTLC-pack. The parameters are loaded from a disk with key (1); key (2) changes the PATH command, key (3) selects scanning parameters, key (4) displays integration parameters. Some of these parameters are:

Spot width: used for recognition of spots on a plate; too narrow peaks are rejected and too broad peaks are used as a baseline.

Threshold: determines a noise limit and mode of integration. Integration is performed by calculation of the first derivative, peaks positions are found and the baseline is constructed. Baseline construction can be done with two different modes;

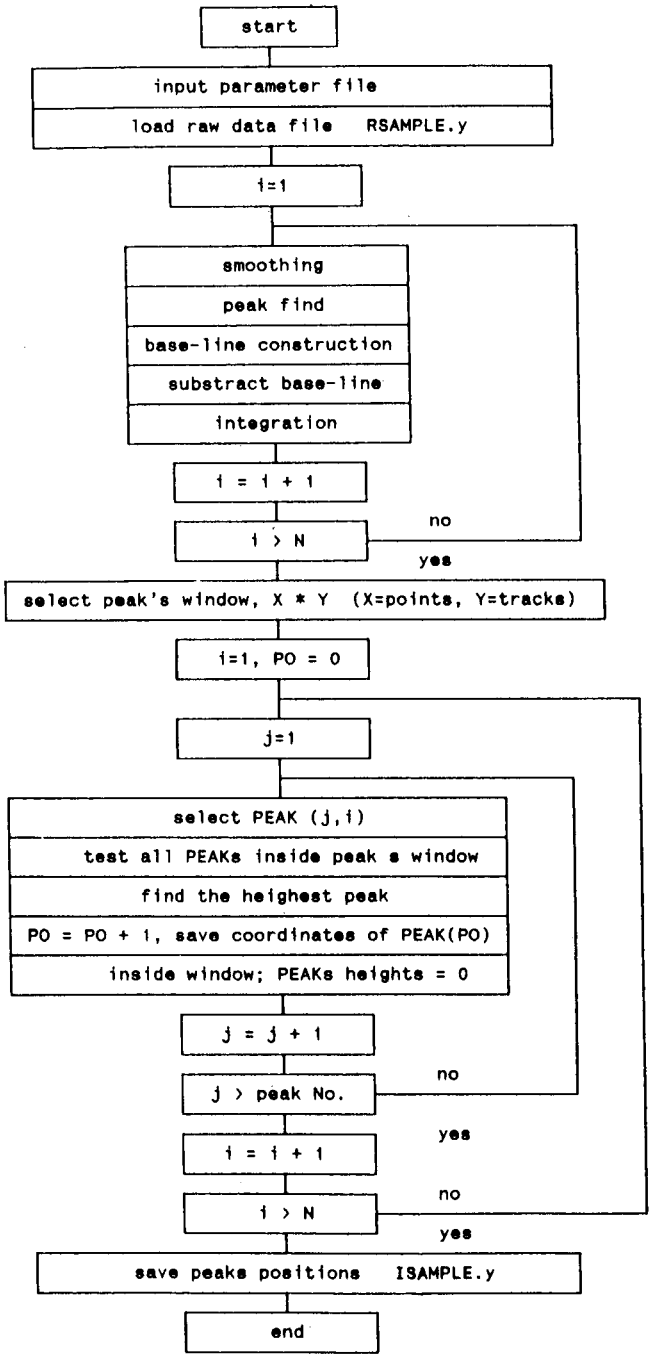

Fig. 3. The flow cart of the scanning program, TDSCAN.COM.

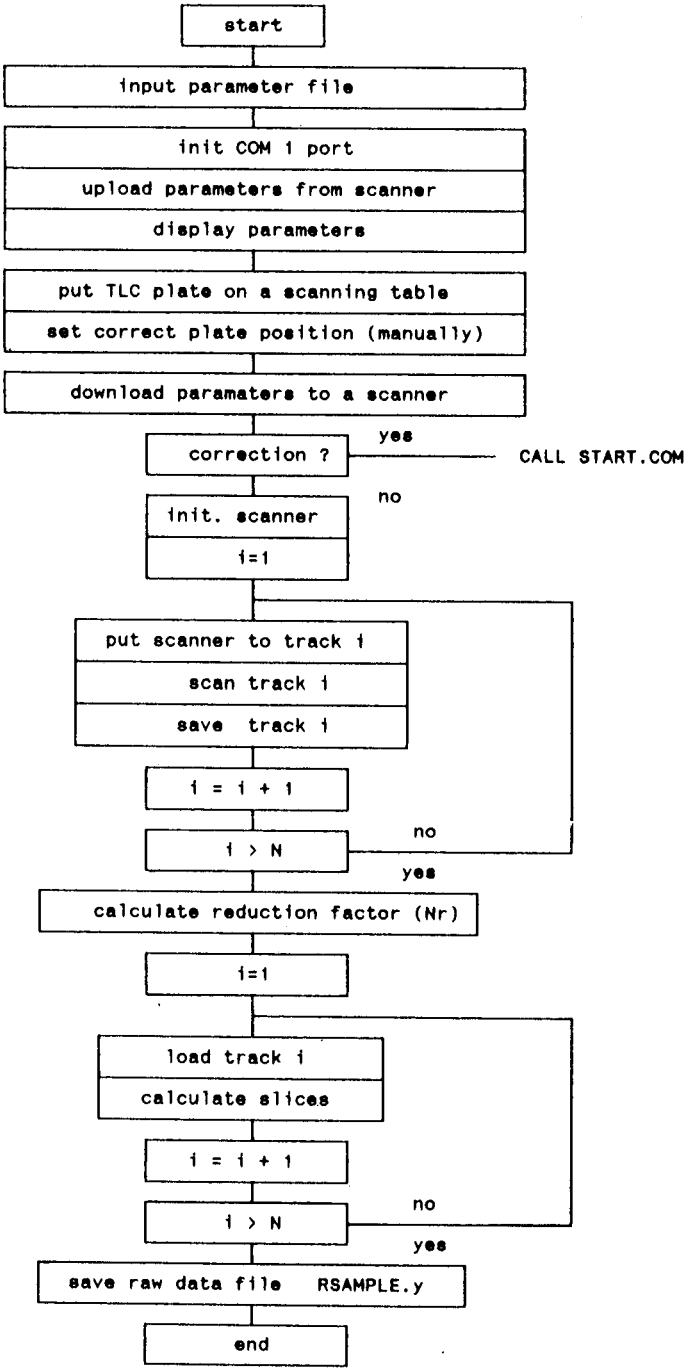

Fig. 4. The flow cart of the integration program, TDINT.COM.

the lowest slope is taken as baseline or a special INTAL algorithm [7] which tests the shapes and positions of peaks beside the slope in order to select the right baseline.

Plot each lane: during the intergration each chromatogram and peak positions can be displayed. This option is useful for examination of integration, but is time consuming.

Print report: can be generated.

Plot: can be generated.

Attenuation: determines the signal level which is used in plotting routine on a screen. If the signal is higher than attenuation a colored point is plotted on the CRT.

Integ. start/from lane: sets the point (mm) from which integration starts in the chromatogram and the lane from which the chromatograms are processed.

Integ end/to lane: sets the point (mm) where the integration is concluded, and selects which track is the last one to be processed.

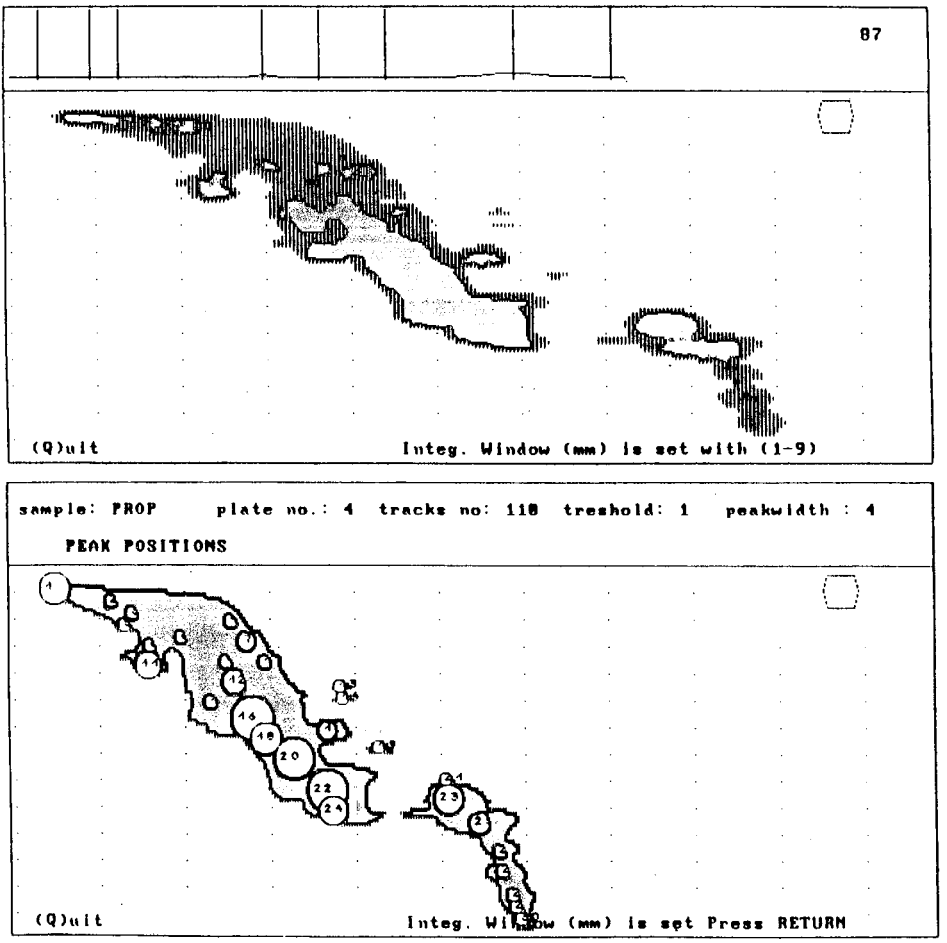

Fig. 5. Presentation of results after integration. The sample is anethanolic extraction of propolis.

Smoothing: during integration, the smooting algorithm can be used according to the selected value.

With key (5) the user program can be linked into the pack.

With key (6) data are stored. It is not possible to store empty files or leave this program without saving data.

The SCAN program collects data from a plate and stores them on a disk; the flow chart for this is shown in Fig. 3. Raw data are stored under RSCAN.X, where X represents a scan number from 1 to the selected the number of scans. This program controls a scanner through the RS232 communication protocol. Commands are English words in ASC format, like START:, RUN:, LOCAL:, INLINE:, etc. Some of these commands are publicly available and documented, some are used only by CAMAG. Scanning parameters are taken from the QLTC.PAR file, and are displayed. The operator loads a TLC plate on a scanning table and sets the plate in the correct starting position using commands on the scanner. The table coordinates are unloaded from a scanner to a PC and the scanning parameters are downloaded. After initializing, the densitometer returns all active parameters which are displayed on a screen.

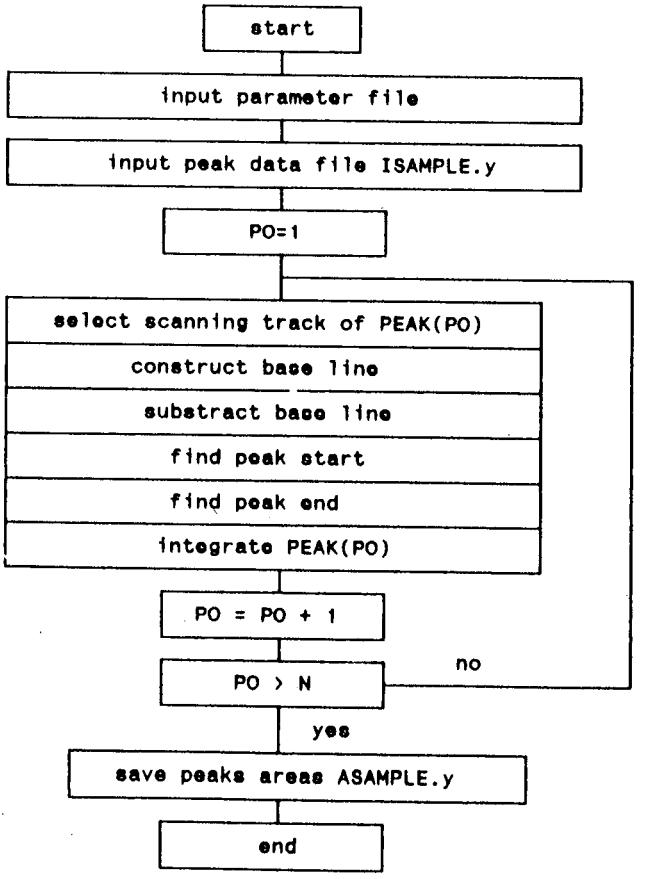

Fig. 6. The flow chart of the reintegration program, TDAREA.COM.

If an error is located, a message describes the type of the error on a screen. The error must be corrected and the initializing of the scanner repeated. After the correct set up a scanning procedure is started. Data are scanned and stored on a disk. After scanning, raw data points are collected into slices. The number of measurements is determined up to a total number of slices less than 360. All slices and plate parameters are stored in one data file.

The TDINT program finds the peak height of each spot on a two-dimensional chromatogram (Fig. 4). On CRT two windows are constructed. In the upper, smaller window, the chromatogram with the constructed baseline is plotted. In the bigger window, the two-dimensional chromatogram is shown. Signals in the x and y directions are shown in different colors, according to the level of the selected attenuation parameters. During the plot the computer calculates the maximum peak of each detected spot and shows it on a screen in the form of circles (Fig. 5). It is possible to add new spots or to delete some already detected peaks with a cursor which is shown on a screen. With left, right, up, and down arrows a cursor is moved across the plate on CRT. With the key (F1) a new spot is fixed, with key (F2) one spot is eliminated, and with (F10) the program is ended.

A special program named TDMAN can be used along with manual correction of the plate already integrated. This program shows a picture of TLC plate on a screen and with a cursor and selected keys it is possible to select one peak, set base line, peak start and peak end. Peak area and position of a peak are shown on the screen and stored on a disk for further processing.

Peaks detected with TDINT are reintegrated with the program TDAREA; the flow chart is shown in Fig. 6. Data are taken from the file ISAMPLE.y. From R_F (x) the successive number of chromatogram in which the maximal peak response is found, and from R_F (y) values the precise positions of the peaks in the selected chromatogram are obtained. The program calculates baseline and finds the start and end of the selected peak (Fig. 7). In this operation the peak width parameter is very important because integration cannot be performed outwith a range of three times

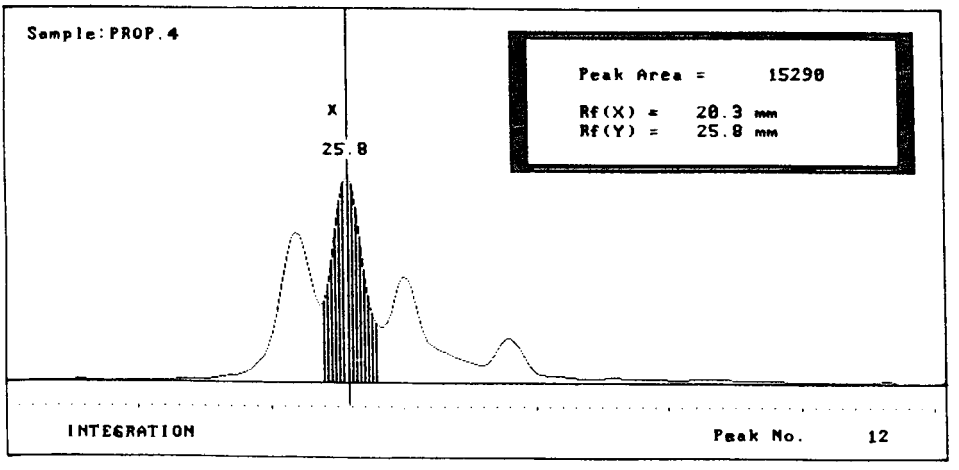

Fig. 7. Reintegration of lanes in which peaks positions were recognised with integration program.

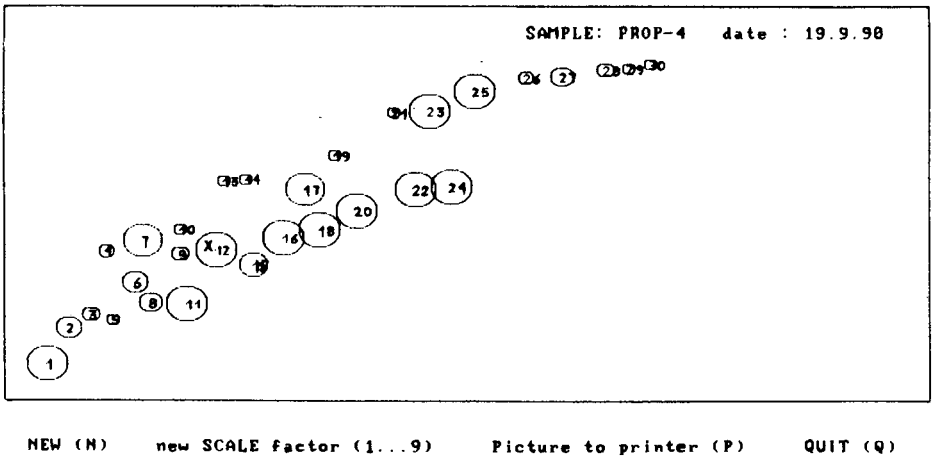

Fig. 8. Documentation of integrated peaks in a two-dimensional chromatogram.

larger than the peak width selected. Integration is documented and printed out. Results are stored in a file named ASAMPLE.y.

Integrated values are documented as two-dimensional chromatograms with the TDDOC program. Peak heights from ISAMPLE.y file or peak areas from ASAMPLE.y file are presented. According to the observed values of R_F (x) and R_F (y) circles are plotted. The diameters of circles correspond to the calculated peak heights or areas (Fig. 8).

TWO-DIMENSIONAL SEPARATION OF PROPOLIS

A good example of integration of td/QTLC-pack is quantitative evaluation of

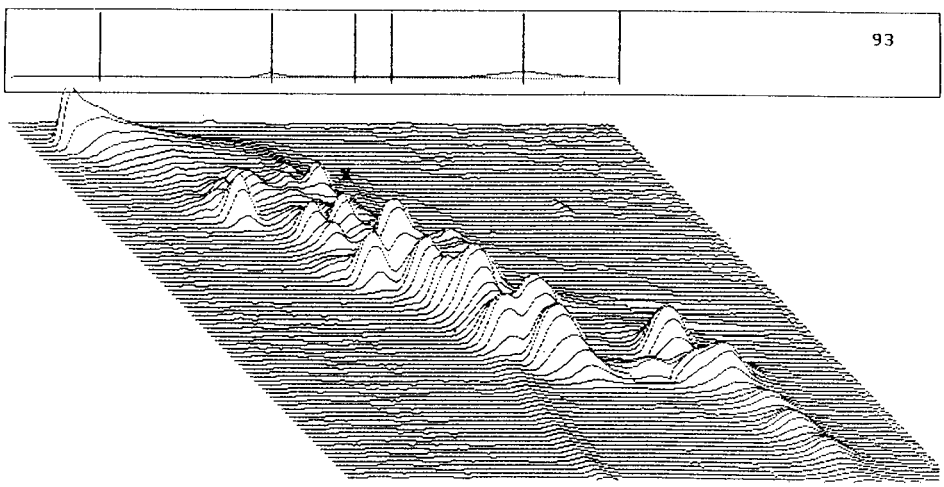

Fig. 9. Three-dimensional presentation of collected data, after substraction of the base line in each lane.

some components in propolis (Fig. 9). Propolis is a very complex mixture of various closely related natural compounds and is therefore an ideal sample for two-dimensional separation. It has been suggested that its biological activity depends mainly on the presence of flavonoids and their content.

Separation of the main phenolic components of the ethanolic extract of propolis was performed on silica 60 F_{254} HPTLC plates. The plates were first developed with the solvent system toluene–ethyl acetate–formic acid (75:15:3), and the development was repeated with the solvent system chloroform–methanol–formic acid (75:5:4) in the perpendicular direction.

Identification and quantification of some characteristic flavonoids was performed using external standards and the 2-aminoethyldiphenylborinate/polyethylene glycol 4000 derivative reagent. Identification and quantitative results of propolis will be given in another paper. Standard deviation of measurements using internal standard and the manual integration mode were $< 5\%$ for substances not separable with linear TLC.

CONCLUSIONS

More information in the quickest possible time is the demand of modern analytical methods. In future TLC will disappear if it cannot provide more information to the user. The main problem in TLC is not application and separation but data collection. Skilled analysts are capable of obtaining much useful information from a plate quite radiply but this information is only qualitative; when they require quantitative results they must use obsolete scanning densitometers in order to obtain values which are only a poor reproduction of data stored on a plate. A computer-controlled scanner is a small step forward, which gives the analyst access to a bigger amount of information, yet such data acquisition is slow. It is useful for two-dimensional TLC, two-dimensional electrophoresis and a combination of both in quantitative determination of amino acids. All these techniques were tested in our laboratory with good results. The described scanning and evaluation system is merely a tool more or less suitable for certain samples. It provides a link between classic linear densitometry and video-oriented data acquisition and image processing of TLC plates.

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CHROMSYMP. 2102

Application of high-performance thin-layer chromatography and automated multiple development for the identification and determination of pesticides in water

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ABSTRACT

The combination of high-performance thin-layer chromatography (HPTLC) with automated multiple development (AMD) allows full automation of the separation step. This provides both a separation efficiency that is considerably better than that in conventional TLC and reproducible gradient elution on the thin layer. Reliable identification of trace amounts of pesticides (*e.g.*, 28 ppt of atrazine) in subsoil, surface and drinking water is clearly demonstrated by AMD-HPTLC using a polarity gradient based on dichloromethane. A second universal elution gradient with changed selectivity on which the substances have different relative migration distances, identification by the multi-wavelength response correlation and the option of recording UV spectra *in situ* constitute three independent methods for reliable and rapid verification. Using 100- instead of 200-μm HPTLC silica gel layers and reducing the running distance increments from 3 to 2 or 1 mm increase the sensitivity, the linearity and the speed of the method.

INTRODUCTION

The identification and determination of trace amounts of pesticides in subsoil, surface and drinking water is required by environmental protection legislation and is of great practical importance. The German drinking water guidelines have been adapted to those of the EC (European Community). They became effective on October 1st, 1986, and their practical implementation has proved a severe challenge to the supervisory authorities.

The tasks confronting these authorities become clearer when one considers the variety of substances to be determined and the low levels of active ingredients allowed per litre of water (0.1 μg for a single substance and 0.5 μg for the sum of all). A multi-sample method is very important, as usually a large number of samples containing many components have to be analysed. The requirements are met by the automated multiple development high-performance thin-layer chromatographic (AMD-HPTLC) method [1]. AMD does not require sample derivatization as do other methods such as gas and high-performance liquid chromatography.

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A multi-sample method of analysing multicomponent samples, using HPTLC and two polarity gradients for AMD, was described by Burger [1]. Burger [2], Weber [3,4] and Zietz and Ricker [5] used HPTLC silica gel plates, generally 200-μm thick and with 3-mm step increments for the polarity gradient. In a systematic investigation [6,7] using standard mixtures, a significant increase in the sensitivity of densitometric detection was obtained with AMD and thinner layers (100 μm).

This paper demonstrates the influence of reducing the layer thickness to 100 μm in combination with reducing the AMD step increments from 3 to 1 mm. The results of the chromatographic analysis of different samples with varying matrix contaminations and smaller amounts (ppt range) of active ingredients are presented. Reference is also made to the interdependence between the parameters described above and the linearity of the calibration graph. These parameters increase if the layer thickness and development step increments are reduced. To obtain an improved chromatographic resolution, the polarity of the first gradient [2,5,6] was optimized in accordance with ref. 5. The optimized method uses silica gel HPTLC plates of layer thickness 100 μm, running distance increments of 2 mm and a 25-step elution gradient containing ten isocratic steps at the start of chromatographic development.

EXPERIMENTAL

Reagents

The identification standards atrazine, desethylatrazine, desisopropylatrazine, isoproturon, terbutylazine, phenmedipham, metamitron, carbetamide, chloridazon, chlortoluron, carbofuran and metribuzin, all of ultra-pure quality, were obtained from Riedel-de Haën (purity at least 99%).

Ammonia solution (25%), concentrated hydrochloric acid, formic acid, acetonitrile (No. 30, gradient grade), *n*-hexane, *n*-heptane, *tert.*-butyl methyl ether (No. 1995 for residue analysis), methanol, dichloromethane and granulated activated carbon (No. 2518) of analytical-reagent grade were purchased from Merck.

Merck 60 F 254 s precoated HPTLC plates (20 × 10 cm) with a 200-μm standard layer thickness and plates from a test batch of the same material but with a 100-μm layer thickness (No. 11764) were used.

Solid-phase extraction glass cartridges, C_{18} (6 ml), silica gel (3 ml), were purchased from J. T. Baker.

It is essential that all reagents are of high quality and that the water is ultra-pure (deionized and membrane filtered).

Instruments

A Linomat IV, an AMD system, a TLC Scanner II with an IBM-compatible computer and CATS evaluation software, including multi-wavelength evaluation, were used. All instruments and software were supplied by Camag.

Sample preparation

C_{18} cartridges were conditioned by immersing them in 10 ml of acetonitrile and applying a vacuum to remove any air bubbles. They were then washed with 100 ml of ultra-pure water (adjusted to pH 2 with HCl). The cartridge was connected to a 1000-ml bottle containing the water sample (adjusted to pH 2 with HCl). The

connection was all glass (plastic tubing must not be used). The sample reservoir vent was connected to a small column packed with activated carbon. A flow-rate of 1–6 ml/min was maintained by means of a peristaltic pump connected to the cartridge outlet. A 1000-ml sample was percolated, then the cartridge was connected to a column packed with activated carbon and air was drawn through for 30 min by means of a water pump. The cartridge was then eluted with 3 ml of acetonitrile.

If the eluate from the C_{18} cartridge had a pronounced yellow colour (humic acid), it was percolated through a silica gel cartridge (1 ml) that had been conditioned with acetonitrile. This cartridge was eluted with 3 ml acetonitrile and washed with a further 1 ml of acetonitrile.

The eluate, either from the C_{18} cartridge direct, or the purified eluate from the silica column, was evaporated to dryness in a rotary evaporator at 35°C under nitrogen. The residue was dissolved in 0.2 ml of acetonitrile–*n*-heptane (95:5) and the solution obtained was used for chromatography.

Calibration and identification standards

All eleven pure substances (12.5 mg of each) were each dissolved in 25 ml of acetonitrile (11 stock solutions of 500 ng/μl). Standard mixtures were prepared as follows: 1 ml of an appropriate stock solution was added to and diluted to 20 ml with acetonitrile. A 1-μl volume of a mixture contains 25 ng of each compound.

The spring water was used as a sample for the determination of its content of atrazine and the metabolite desethylatrazine. The following two standard mixtures are routinely in use: mixture A, 1 ml of metamitron, 1 ml of carbetamide, 1 ml of chloridazon, 1 ml of chlortoluron, 1 ml of atrazine and 1 ml of carbofuran; and mixture B, 1 ml of desisopropylatrazine, 1 ml of desethylatrazine, 1 ml of isoproturon, 1 ml of terbutylazine and 1 ml of phenmedipham.

Preparation of spiked water samples and sample application

A 200-μl volume of each of the standard mixtures was added to 1 l of ultra-pure water, which corresponds to a 5 ppb level of each of the compounds present. These water samples were extracted as described under *Sample preparation*. HPTLC plates were prewashed by immersion for 1 h in isopropanol, followed by drying for 60 min at 120°C. Sample application was performed by the spray-on technique using a Linomat IV. Volumes of 10 μl each of the standard mixtures (A and B above), 10–80 μl of the extracts from the water samples and 10 μl of a blind extract (from non-spiked water) were applied as 8-mm bands, 4 mm apart; the delivery rate was 10 s/μl.

Chromatogram development

The chromatographic plates were developed using the AMD system with the 25-step universal gradient depicted in Fig. 1. This gradient, based on dichloromethane, is considered to be a screening gradient. In the event of a positive result, a second gradient based on *tert.*-butyl methyl ether is used for confirmation.

Prior to actual gradient development, the plate was developed for a distance of 8 mm with ten alkaline isocratic steps using pure acetonitrile, in order to move all soluble material away from the insoluble matrix at the area of sample application.

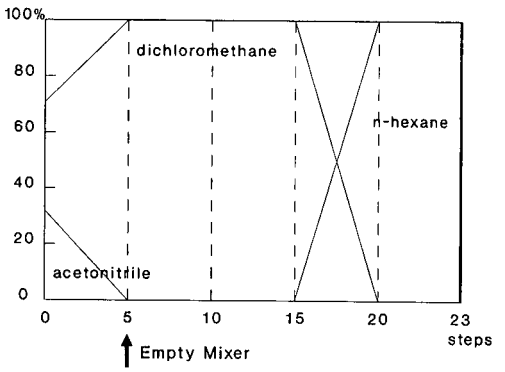


Fig. 1. The AMD elution gradient, designed for screening based on dichloromethane.

Densitometric evaluation

The underivatized plates were scanned by measuring the absorbance at six or seven different wavelengths in the TLC Scanner II using CATS evaluation software with the multi-wavelength option. The scanning parameters were a deuterium lamp, slit dimensions 0.2 × 5 mm, monochromator band width 10 nm and wavelengths 190, 200, 220, 240, 260, 280 and 300 nm; a multi-colour plotter was used for the graphical presentation of the analogue curves (coloured versions of Figs. 2–4 below are available on request). Using these scanning conditions and flushing the optical unit with nitrogen, each substance was quantified by the absorbance very close to its maximum response wavelength.

RESULTS AND DISCUSSION

The overall recoveries were determined on spiked, contaminant-free water and found to be 70% for metamitron, 79% for carbetamide, 95% for chloridazon, 88% for chortoluron, 98% for atrazine, 66% for carbofuran, 63% for desisopropylatrazine, 85% for desethylatrazine, 97% for isoproturon, 93% for terbutylazine and 92% for phenmedipham.

Fig. 2 shows multi-wavelength scans of the standard mixtures A and B. It can be seen that the identification standards are well resolved; they can be reliably identified by their multi-wavelength response correlation. An attempt to resolve all eleven substances on one track and identify them reliably was abandoned. Depending on the relative migration distances, many other combinations can be assembled into one group if screening for a certain selection is intended.

The analysis of a real sample of spring water is depicted in Fig. 3. Compounds identified were 72 ng/l (ppt) of atrazine (38.5 ng absolute), 94 ng/l of the metabolite desethylatrazine (50 ng absolute), 67.5 ng/l of chlortoluron (36 ng absolute) and 178 ng/l of 2,4-DP (dichlorprop) (95 ng absolute). These values were found with samples containing comparatively large amounts of humic acid. In the absence of humic acid, lower values can be expected.

The low-level detectability is shown in Fig. 4. The small peaks in the sample of drinking water are reliably identified as 28 ng/l of atrazine (7.5 ng absolute) and 43 ng/l of desethylatrazine (9.3 ng absolute).

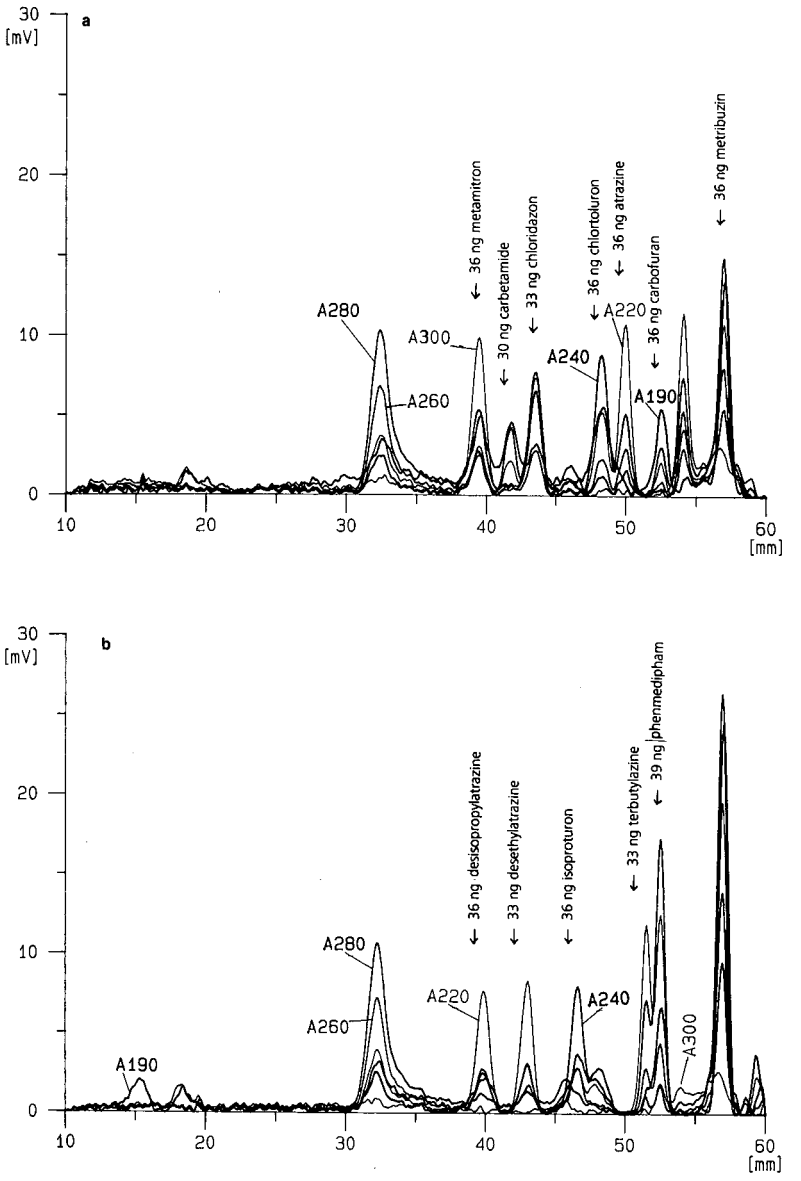


Fig. 2. Multi-wavelength scans of (a) standard mixture A and (b) standard mixture B, scanned at six different wavelengths (190–300 nm) with the plots superimposed. (Original, coloured plots of Figs. 2–4 are available from the authors upon request).

The influence of reducing the layer thickness from 200 to 100 μm on sensitivity is apparent from Table I and Fig. 5. The signals can be increased by a factor of 2.5 or more by reducing both the layer thickness and step increments. The linearity of the calibration graph and the dependence on the layer thickness and AMD step increments

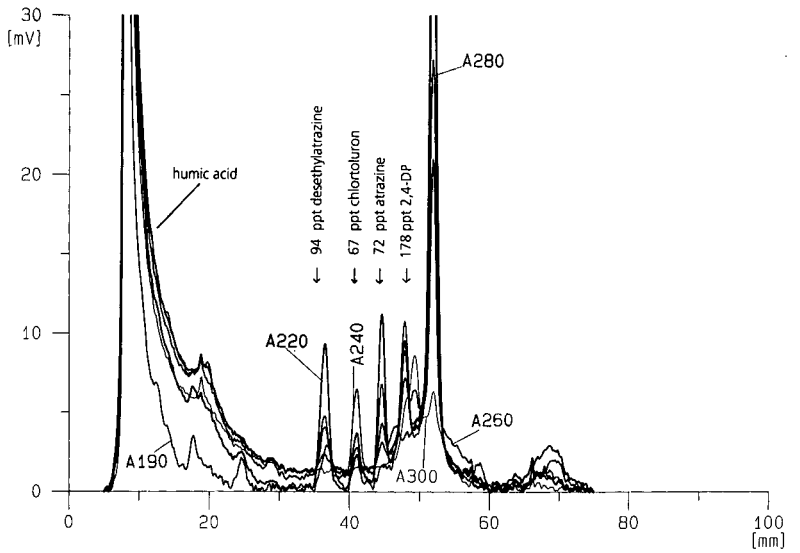


Fig. 3. Multi-wavelength scan of a spring water sample containing 72 ppt of atrazine, 94 ppt of desethylatrazine, 67 ppt of chlortoluron and 178 ppt of 2,4-DP (dichlorprop). The sample was scanned at six different wavelengths (190–300 nm) and the plots are superimposed.

are depicted in Fig. 6. Thinner layers and short step increments correlate with improved linearity of the peak-area (height) integrals *versus* concentration relationship.

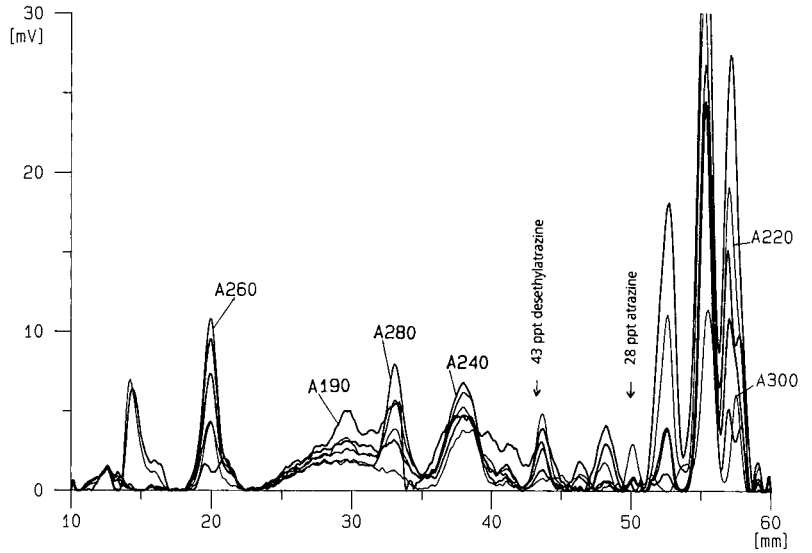


Fig. 4. Multi-wavelength scan of a drinking water sample containing 28 ppt of atrazine and 43 ppt of desethylatrazine.

TABLE I

RESPONSE AS ABSORBANCE AT 240 nm, MEASURED ON 200- AND 100-μm PLATES CHROMATOGRAPHED WITH DIFFERENT STEP INCREMENTS (1,2 and 3 mm)

The inconsistent ratios of the response under different conditions is believed to be due to the fact that some of the compounds had their absorption maximum away (up to 35 nm) from the measuring wavelength.

Component		Layer thickness (μm)					
		200			100		
		Step increment (mm)					
		3	2	1	3	2	1
Metoxuron	1	100	144	167	288	300	322
Monuron	2	100	133	167	267	292	308
Chlortoluron	3	100	125	158	250	267	275
Cyanazine	4	100	121	129	186	221	257
Propazine	5	100	84	100	142	179	179
Vinclozoline	6	100	82	75	163	138	150

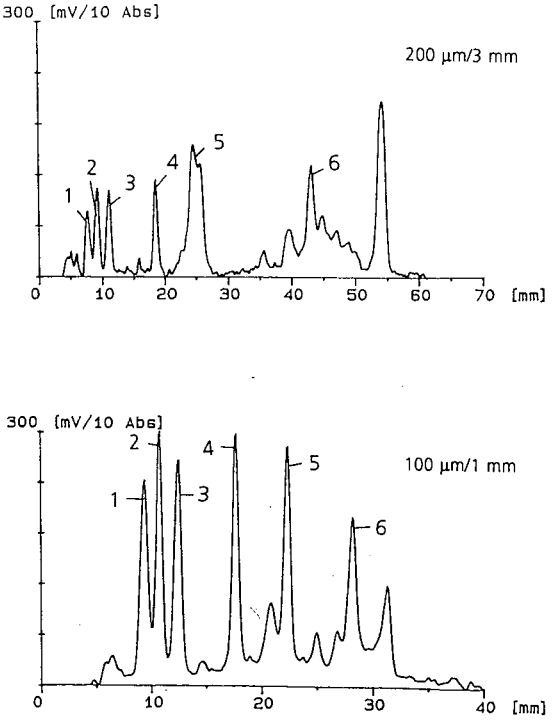
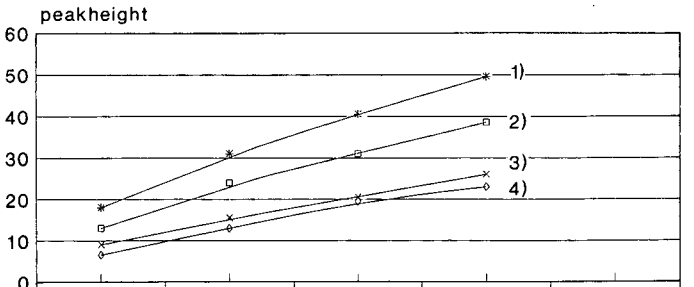


Fig. 5. Sensitivity and dependence on layer thickness of the mixture detailed in Table I.



		250 ng	500 ng	750 ng	1000 ng	Lin. Reg.
1)	100/1	18	31	40.5	49.5	y = 0.0408x +9.0
2)	100/3	13	24	31	38.5	y = 0.0334x +5.75
3)	200/1	9	15.5	20.5	26	y = 0.0224x +3.75
4)	200/3	6.5	13	19.5	23	y = 0.0224x +1.5

Fig. 6. Influence of the layer thickness and step increment of the AMD development gradient on the linearity of the calibration graph. 100/1, etc. = layer thickness (μm)/step increment (mm); the equations under Lin. Reg. are the calculated linear regressions of the calibration functions in the range of 250–1000 ng cyanazine; the values under 250–1000 ng are peak heights.

ACKNOWLEDGEMENTS

The authors are greatly indebted to Birgit Knehr and Gerhard Keller of the Untersuchungs- und Forschungslabor Umwelt (Herbrechtingen, Germany) for valuable assistance with the samples.

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CHROMSYMP. 2191

Problems and solutions in chiral thin-layer chromatography: a two-phase "Pirkle" modified amino-bonded plate

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ABSTRACT

The preparation of a two-phase "Pirkle" modified amino-bonded thin-layer chromatography (TLC) plate for enantiomer separation is described. The plate was prepared by partially immersing a commercially prepared aminopropyl high-performance TLC plate in a solution of the chiral selector N-(3,5-dinitrobenzoyl)-L-leucine. The portion of the plate modified with the chiral selector was used to separate the enantiomers of the model compounds 2,2,2-trifluoro-(9-anthryl)ethanol and 1,1'-binaphthol. The separated enantiomers were then eluted, using continuous development onto the unmodified portion of the plate. The absence of the chiral selector on this segment of the phase allowed the detection of the separated enantiomers by fluorescence quenching.

INTRODUCTION

The availability of cheap, reliable, robust and efficient methods for the resolution of enantiomers by thin-layer chromatography (TLC) would represent a valuable addition to the high-performance liquid and gas–liquid chromatographic methods currently available. However, at present enantiomer resolution by TLC is still poorly developed. Indeed the only type of separation to be commercialised is that based on ligand exchange [1,2] and whilst these plates give excellent results [3,4] they are limited in the range of compounds for which they can be used. Some success has also been achieved using cyclodextrins, and their derivatives, as mobile phase additives [5,6] or cyclodextrins chemically bonded to silica gel [7]. Ion-pair reagents such as N-benzoxycarbonylglycyl-L-proline and related materials [8,9] and champhor sulphonic acid [9] have also been employed as mobile phases additives in order to effect chiral separations.

The use of "Pirkle"-type stationary phases in TLC represents another obvious method for enantiomer separation, and indeed such plates have been described by several groups [10,13]. A potential disadvantage of this type of phases is the very high

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UV background due to the chiral selector (*e.g.*, dinitrobenzoylphenylglycine or dinitrobenzoylleucine) which limits their use to fluorescent or coloured materials.

As we describe here, this limitation can be overcome by the use of two-phase plates where one portion of the plate is modified with the chiral selector to obtain the separation and the remainder left untreated to enable detection.

EXPERIMENTAL

Test compounds and reagents

2,2,2-Trifluoro-(9-anthryl)ethanol, (*R*)-(+)-1,1′-bi-2-naphthol and (*S*)-(−)-1,1′-bi-2-naphthol were obtained from Aldrich (Gillingham, U.K.). The chiral selector, N-(3,5-dinitrobenzoyl)-L-leucine, was purchased from Sigma (Poole, U.K.) Solvent were of HPLC grade and were obtained from BDH (Poole, U.K.).

TLC plates and treatments

Amino-bonded high-performance (HP) TLC plates (10 × 10 cm, glass backed, E. Merck, Cat. No. 15647) were purchased from BDH. Plates were prepared by immersing the portion of the plate to be modified (5–6 cm) into a 0.05 *M* solution of N-(3,5-dinitrobenzyl)-L-leucine in tetrahydrofuran (THF) for a few seconds. The plate was then immersed in THF to remove any unbound chiral selector and left to dry in a fume cupboard at ambient temperature.

Chromatography

Samples (0.5–20 μg) of the test compounds were applied to the treated plates as solution (~1 mg/ml) in methanol either as spots using 1-μl glass capillaries or as 1-cm streaks using a Camag Linomat IV TLC sample applicator (Camag, Switzerland). The plates were then subjected to continuous chromatography, with the solvent allowed to migrate up to approximately 8 cm before evaporation, using mixtures of 2-propanol–hexane as solvents (see text for details).

Following chromatography compounds present on the plate were detected visually as yellow spots if present on the Pirkle phase or by fluorescence quenching at 254 mm if present on the untreated portion of the plate. Scanning densitometry was performed using a Shimadzu CS 9000 scanning densitometer. For 2,2,2-trifluoro-(9-anthryl)ethanol the wavelengths used for scanning densitometery were 380 and 230 nm for the Pirkle and untreated portions of the plate, respectively. For bi-2-naphthol the wavelengths employed for scanning densitrometry on the Pirkle phase and the untreated portion of the plate were 328 and 230 nm, respectively. These wavelengths were chosen based on *in situ* UV absorbance spectra obtained on the compounds of interest.

RESULTS AND DISCUSSION

In order to demonstrate the principle of these two-phase plates for the separation and subsequent detection of enantiomers the model compounds 2,2,2-trifluoro-(9-anthryl)ethanol and 1,1′-binaphthol were chosen. A particular advantage of these compounds is that they can be visally detected on the Pirkle modified portion of the plate [4,10–13] (albeit at lower sensitivity than on the untreated segment of the

plate). This enabled enantiomer separation to be optimised prior to elution onto the untreated "detection zone". Clearly, with compounds which are not visible on the Pirkle phase method development will be more protracted.

We have used both N-(3,5-dinitrobenzoyl)-*R*-(−)-α-phenylglycine and L-leucine for the preparation of ionically coated Pirkle plates [4,13]. In our hands the L-leucine analogue, when used to modify commercially prepared aminopropyl-bonded TLC plates, gave the best results for the separation of 2,2,2-trifluoro-(9-anthryl) ethanol. In the studies described here Pirkle plates were prepared very simply by dipping aminopropyl-bonded HPTLC plates into a solution of (*R*)-N-(3,5-dinitrobenzoyl)-L-leucine (0.05 *M*) in THF. In Fig. 1A a typical separation of (±)-2,2,2-trifluoro-(9-anthryl)ethanol enantiomers on such a plate is shown. This separation was obtained using *n*-hexane–2-propanol (80:20, v/v) as solvent. The separated enantiomers could be detected visually as yellow spots when present on the Pirkle phase. Scanning densitometry gave the relative proportions of the separated enantiomers to be 47 and 53% for the (−) and (+) forms, respectively.

In Fig. 1B a similar separation of (*R*)-(+)-1,1′-bi-2-naphthol and (*S*)-(−)-1,1′-bi-2-naphthol is illustrated, These substances were also readily detected as yellow spots on the Pirkle phase. This separation was achieved using *n*-hexane-2-propanol (20:80, v/v) as solvent. Once again scanning densitometry allowed the relative proportions of the two components to be determined as 50.2 and 49.8% for the (+) and (−) enantiomers, respectively.

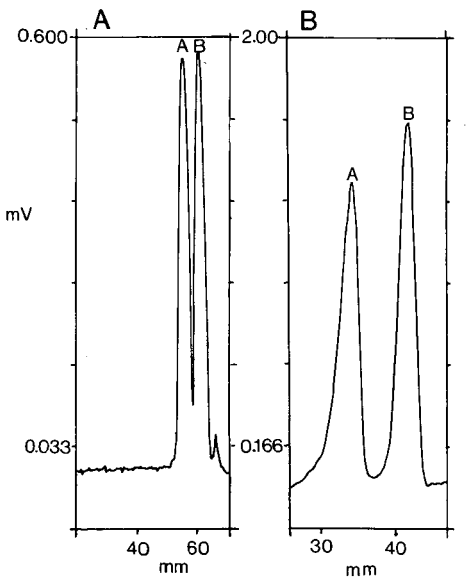

Fig. 1. (A) Separation of the enantiomers of 2,2,2-trifluoro-(9-anthryl)ethanol (peaks A and B) on N-(3,5-dinitrobenzoyl)-L-leucine ionically bonded to aminopropyl-bonded silica gel HPTLC plates using *n*-hexane–2-propanol (80:20, v/v) as solvent. Scanning densitometry was performed at 380 nm. Spots appeared yellow on a pink background. (B) Separation of (*R*)-(+)-1,1′-bi-2-naphthol (peak A) and (*S*)-(−)-1,1′-bi-2-naphthol (peak B) on N-(3,5-dinitrobenzoyl)-L-leucine ionically bonded to aminopropyl-bonded silica gel HPTLC plates using *n*-hexane–2-propanol (20:80, v/v) as solvent. Scanning densitometry was performed at 328 nm. Spots appeared yellow on a pink background.

Having obtained these separations, plates were then prepared with only the lower portion of phase modified with the N-(3,5-dinitrobenzoyl)-L-leucine. The enantiomers of both 2,2,2-trifluoro-1-(9-anthryl)ethanol and 1,1′-bi-2-naphthol were separated on the lower, Pirkle portion of the plate and then eluted into the untreated region using continuous development.

Multiple development techniques were also investigated but were not as effective as continuous development.

The results of these experiments are shown in Fig. 2A and B. These chromatograms show only results for the untreated portion of the plates.

The separated enantiomers of 2,2,2-trifluoro-1-(9-anthryl)ethanol are shown in Fig. 2A. Excellent resolution was obtained, with scanning densitometry giving the relative proportions of the separated components to be 48.5 and 51.4 for the (−) and (+) enantiomers, respectively. As can been seen from the chromatogram the "solvent front" (*i.e.* the point at which the solvent was allowed to evaporate) was marked by the presence of significant UV absorbance. This interference appears to have been due to the leaching out of the chiral selector (or a degradation product) from the Pirkle portion of the plate.

The separation of the binaphthol enantiomers on the Pirkle phase followed by migration onto the untreated detection zone of these two-phases plates is shown in Fig. 2B. Once again excellent separation of the two-enantiomers was achieved. However, in the particular example illustrated in the figure the continuous development

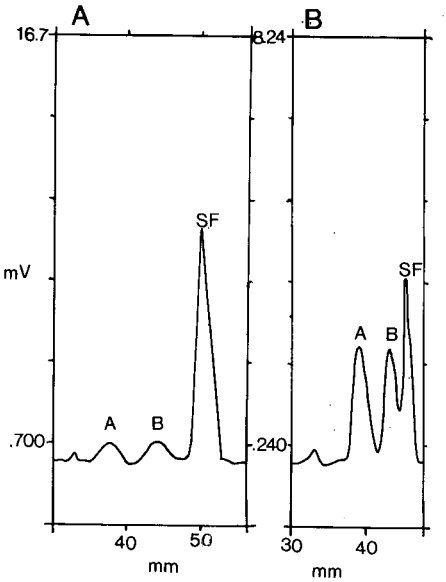

Fig. 2 (A) Separation of the enantiomers of 2,2,2-trifluoro-(9-anthryl)ethanol (peaks A and B) on aminopropyl-bonded HPTLC plates partially coated with N-(3,5-dinitrobenzoyl)-L-leucine (see Fig. 1A for solvents). Scanning densitometry was performed at 230 nm. Only the uncoated portion of the plate is shown. SF-solvent front. (B) Separation of (*R*)-(+)-1,1′-bi-2-naphthol (peak A) and (*S*)-(−)-1,1′-bi-2-naphthol (peak B) on aminopropyl-bonded HPTLC plates partially coated with N-(3,5-dinitrobenzoyl)-L-leucine (see Fig. 1B for solvents). Scanning densitometry was performed at 230 nm. Only the uncoated portion of the plate is shown. SF = solvent front.

was continued for too long with the result that the fastest moving (−) enantiomer had begun to merge with the interfering Pirkle phase-related material running at the solvent front. This example is shown to emphasise the importance of careful optimisation of (a) the relative proportions of the Pirkle and detection zones of the plate and (b) of the length of time employed for continuous development once the enantiomers have been eluted from the Pirkle phase.

Subsequent separations (not shown) with a larger proportion of the plate left unmodified with the selector, giving a larger detection zone, eliminated the problem highlighted by Fig. 2B.

In the configuration described here the lower portion of the plate was modified with the chiral selector and the upper portion left untreated for detection. An alternative configuration, where the separation of the enantiomers requires more of the plate to be modified with the chiral selector, or where the mixture to be separated is more complex, is where only a thin strip (1–3 cm) of the plate is coated with the Pirkle reagent. The enantiomers are separated on this strip of Pirkle phase using the whole length of the plate (with or without continuous development) and the plate is then rotated through 90° and subjected to two-dimensional development to elute the separated components onto the detection zone. Clearly, only one sample can be loaded onto a plate of this configuration.

In order to obtain good results spots rather than streaks must be used reducing the amount of material which can be applied. This type of approach has also been implemented by other workers with some success [14].

CONCLUSIONS

The use of two-phase TLC plates to first separate and then detect enantiomers is a practical and readily implemented solution to the problems posed by the fluorescence quenching of Pirkle chiral selectors. We have found such plates to be robust and reliable, giving good separations of suitable compounds over the range 0.5–20 μg (applied as 1 cm bands). Continuous development gave the best results with this type of plate.

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Chiral separation by cyclodextrin-modified micellar electrokinetic chromatography

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ABSTRACT

Chiral separation by micellar electrokinetic chromatography (MEKC), which permits the separation of uncharged or electrically neutral compounds by the electrophoretic technique, was achieved through the addition of cyclodextrins (CDs) to sodium dodecyl sulphate (SDS) micelle solution. In this cyclodextrin-modified MEKC (CD-MEKC), CDs cannot be solubilized to the SDS micelle and migrate with the same velocity as that of the electroosmotic flow. The solutes are distributed among three phases, an aqueous phase, the micelle and the CD. Chiral recognition depended on the type of CD; in particular, γ-CD was effective for the chiral separation in this method. The addition of an organic solvent or a chiral compound such as sodium *d*-camphor-10-sulphonate or *l*-menthoxyacetic acid to the SDS micelle solution containing CDs improved the enantioselectivity. The addition of CDs reduced the capacity factors of solutes; in contrast, chiral additives increased them. The resolution was optimized by changing the concentrations of CDs and chiral additives. The chiral separation mechanism is also briefly discussed.

INTRODUCTION

Micellar electrokinetic chromatography (MEKC) [1] is a recently developed high-resolution separation method [2–5]. MEKC, which uses the capillary zone electrophoretic (CZE) technique, permits the separation of uncharged or electrically neutral compounds [2,3] and offers greatly improved selectivity for the separation of ionic compounds [4–7].

Stereochemistry can have a significant effect on the biological activity of a drug. Racemic drugs exhibit pharmacological activities and/or side-effects different from those of the optically pure drugs [8]. It is therefore important to develop a chiral separation method for the determination of optical purity. A variety of chromatographic approaches, particularly those using high-performance liquid chromatography (HPLC), have been developed [9]. Recently, much work has been reported on the direct resolution of enantiomers by chiral stationary phases and a wide variety of the stationary phases are now commercially available.

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In MEKC, chiral separation has been achieved by using a chiral surfactant such as bile salts [10–12] or sodium N-dodecanoyl-L-valinate [13,14]. A mixed micelle of chiral surfactants as mentioned above or chiral compounds such as digitonin [13] with achiral sodium dodecyl sulphate (SDS) [12–15] has also been successfully used for the chiral separation of some DL-amino acid derivatives.

The resolution of enantiomers by cyclodextrin-modified MEKC (CD-MEKC) with SDS [16] was investigated in this work. CDs are widely used in analytical applications, especially as a mobile phase additive or a chiral moiety of the stationary phase in HPLC [9]. CDs cannot be solubilized into the SDS micelle employed in MEKC, because of the hydrophilic nature of the external portion. In CD-MEKC the solutes are distributed among three phases, an aqueous phase, the micelle and the CD. Differential inclusion-complex formation of a CD with the solute provides the differential solute migration and chiral recognition. CDs were used successfully for chiral separation in high-performance capillary gel electrophoresis by Guttman *et al.* [17] and in CZE by Fanali [18]. Chiral separation by cyclodextrin EKC, in which cyclodextrin derivatives are employed as a carrier, was reported by Terabe [1].

Chiral separation by high-performance capillary electrophoresis (HPCE), to which both MEKC and CZE belong, is a relatively new technique. There are many advantages of the HPCE mode compared with HPLC. Chiral separation has been achieved easily in the HPCE mode by adding chiral surfactants or chiral compounds, which interact with the enantiomeric solute, to the buffer solution without changing the capillary tube. High resolution is achieved within a short time. HPCE should have other capabilities and advantages for chiral separations which are now being investigated.

This paper describes the chiral separation of enantiomers of some drugs and chemicals by employing five types of CDs with SDS solutions. The effects of CD concentration, organic solvent addition and chiral compound addition on chiral recognition are discussed. A possible chiral separation mechanism is suggested.

EXPERIMENTAL

Apparatus for MEKC

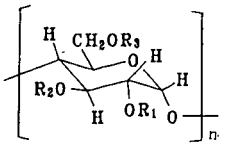
A fused-silica capillary of length 65 cm (effective length 50 cm) and I.D. 0.05 mm (Scientific Glass Engineering, Ringwood, Australia) was used as a separation tube. A Model HJLL-25PO high-voltage d.c. power supply (Matsusada Precision Devices, Kusatsu, Shiga, Japan) delivering from 0 to 25 kV was used to drive the MEKC separation. The migrating solutes were detected by the on-column measurement of UV absorption at 220 nm with an SPD-6A spectrophotometer (Shimadzu, Kyoto, Japan) at a time constant of 0.05 s using a laboratory-made cell holder and a slit. A Chromatopac C-R5A instrument (Shimadzu) was used for data processing. Other apparatus and experimental procedures were the same as described previously [6].

Reagents

SDS and five types of CDs, α-cyclodextrin (α-CD) β-cyclodextrin (β-CD), 2,6-di-O-methyl-β-cyclodextrin (DM-β-CD), 2,3,6-tri-O-methyl-β-cyclodextrin (TM-β-CD) and γ-cyclodextrin (γ-CD), were obtained from Nacalai Tesque (Kyoto, Japan).

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF CYCLODEXTRINS USED



Parameter	α-CD	β-CD	DM-β-CD	TM-β-CD	γ-CD
n	6	7	7	7	8
R_1	H	H	CH_3	CH_3	H
R_2	H	H	H	CH_3	H
R_3	H	H	CH_3	CH_3	H
Molecular weight	973	1135	1331	1430	1297
Cavity diameter (Å)	5–6	7–8	7–8	7–8	9–10

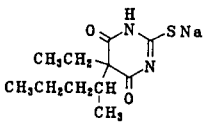
Some physico-chemical properties of CDs are summarized in Table I [19]. Sodium *d*-camphor-10-sulphonate and *l*-menthoxyacetic acid were obtained from Nacalai Tesque. *l*-Menthoxyacetic acid was converted to the sodium salt form by titrating the solution with 1 *M* sodium hydroxide solution. All other reagents and solvents were of analytical-reagent grade from Katayama Kagaku Kogyo (Osaka, Japan). Water was purified with a Milli-RO 60 water system (Millipore Japan, Tokyo, Japan). The micellar solution was prepared by dissolving 0.05 *M* SDS in 0.02 *M* phosphate–borate buffer solution of pH 9.0 and the solution was passed through a membrane filter of 0.45-μm pore size (Gelman Science, Japan, Tokyo) and degassed by sonication with a Branson Model B-2200 ultrasonic cleaner (Yamato, Tokyo, Japan) prior to use.

Five enantiomeric drugs or compounds and two achiral barbiturates were used as test solutes. Thiopental (sodium salt) and pentobarbital (calcium salt) were obtained from the research laboratories at Tanabe Seiyaku (Osaka, Japan). Phenobarbital and sodium barbital were purchased from Wako (Osaka, Japan). 2,2′-Dihydroxy-1,1′-dinaphthyl and 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate were purchased from Aldrich (Milwaukee, WI, U.S.A.). 2,2,2-Trifluoro-1-(9-anthryl)ethanol was purchased from Nacalai Tesque. Chemical structures of the solutes are shown in Fig. 1. The sample solutions were prepared by dissolving each solute in methanol at concentration of *ca.* 1 mg/ml so that adequate peak heights could be obtained. Sudan IV from Aldrich or diltiazem derivative from the research laboratory at Tanabe Seiyaku were used as tracers of the micelle. These solutes migrated last in all the experiments, although these may be partially included in the CD.

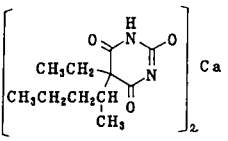
RESULTS AND DISCUSSION

Chiral recognition of CDs

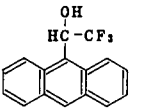
The effect of the type of CD on the chiral recognition of five enantiomeric solutes was first investigated by using a buffer solution of 0.05 *M* SDS and 4 *M* urea, to which each CD was added. Calculated capacity factors ($\tilde{k}'$), according to the



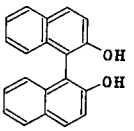
1. Thiopental (sodium)



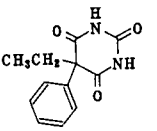
2. Pentobarbital (calcium)



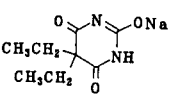
3. 2,2,2-Trifluoro-1-(9-anthryl)ethanol



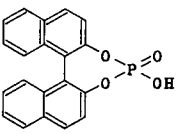
4. 2,2'-Dihydroxy-1,1'-dinaphthyl



5. Phenobarbital



6. Barbital (sodium)



7. 1,1'-Binaphthyl-2,2'-diyl hydrogen phosphate

Fig. 1. Structures of the solutes.

equation in ref. 2, and separation factors (α) are summarized in Table II. The concentrations of CDs were 15 mM for DM-β-CD, TM-β-CD and γ-CD and 40 mM for α-CD and β-CD to give $\tilde{k}' \approx 1$ for two enantiomeric barbiturates. The concentration effects (extent of reduction of migration times) of the former were larger than those of the latter [20].

Chiral recognition of the solutes was observed with four CDs, except for α-CD. Enantiomers of thiopental and of pentobarbital were only resolved by using γ-CD, although their resolution was not sufficient. A typical chromatogram of four enantiomeric solutes using a 0.05 M SDS solution containing 30 mM γ-CD is shown in Fig. 2. Chiral recognition depended on the type of CD. Among the five CDs, γ-CD was the most effective for the chiral recognition of the solutes. These results are

TABLE II

CHIRAL RECOGNITION BY FIVE CYCLODEXTRINS

Buffer, 0.05 M SDS buffer solution of pH 9.0 containing 4 M urea and CDs. Applied voltage, 20 kV. Ambient temperature.

Solute (No.)	α-CD (40 mM)		β-CD (40 mM)		DM-β-CD (15 mM)		TM-β-CD (15 mM)		γ-CD (15 mM)	
	$\tilde{k}'$	α	$\tilde{k}'$	α	$\tilde{k}'$	α	$\tilde{k}'$	α	$\tilde{k}'$	α
Thiopental (1)	0.92	1	0.78	1	1.01	1	1.38	1	1.06	1.03
	–		–		–		–		1.09	
Pentobarbital (2)	0.92	1	0.88	1	1.06	1	1.28	1	1.13	1.02
	–		–		–		–		1.15	
Binaphthyl phosphate (7)	1.49	1	1.19	1.04	2.64	1.09	3.65	1.09	1.45	1.03
	–		1.24		2.88		3.97		1.50	
Dinaphthyl (4)	11.84	1	9.87	1	14.62	1	15.32	1.08	20.06	1.08
	–		–		–		16.51		21.66	
Anthrylethanol (3)	15.05	1	10.94	1.04	16.62	1	48.57	1	23.26	1.48
	–		11.42		–		–		34.32	

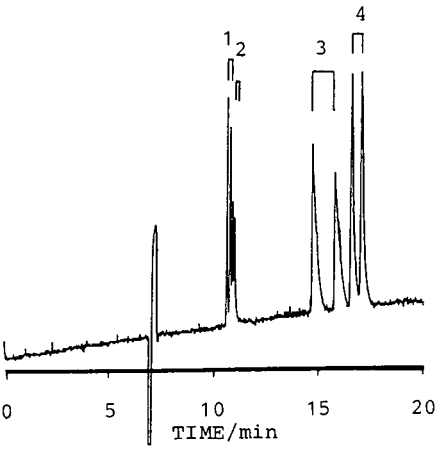


Fig. 2. Chiral separation of four enantiomers. Samples are identified by the same numbers as given in Fig. 1. Conditions: buffer, 0.02 *M* phosphate–borate buffer of pH 9.0 containing 0.05 *M* SDS and 30 m*M* γ-CD; separation tube, 650 mm × 0.05 mm I.D. (effective length 500 mm); applied voltage, 20 kV; detection, 220 nm; temperature, ambient.

different, however, from the results in HPLC using CDs as chiral additives to the mobile phase or as a chiral moiety of the stationary phase [9]. The cavity diameter (fitness of the solute) and hydrophobic nature of the internal portion of CDs probably influenced the effective or differential inclusion-complex formation of the enantiomers, leading to differential migration and successful chiral separation.

In a micellar solution, a monomeric surfactant, which is in equilibrium with the micelle, exists in an aqueous phase and it can be included by the CD because of the presence of the lipophilic hydrocarbon chain. This may prevent the solute from inclusion. CDs having wider cavity such as γ-CD have the capability of including the solute together with the surfactant monomer. A schematic illustration is shown in Fig. 3. The cavity size that is most suitable for chiral recognition is different between

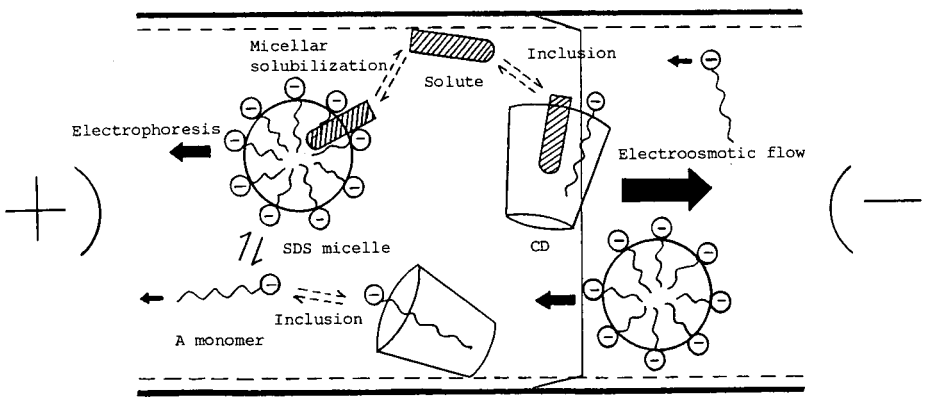


Fig. 3. Schematic illustration of the solute interaction in MEKC using SDS and CD.

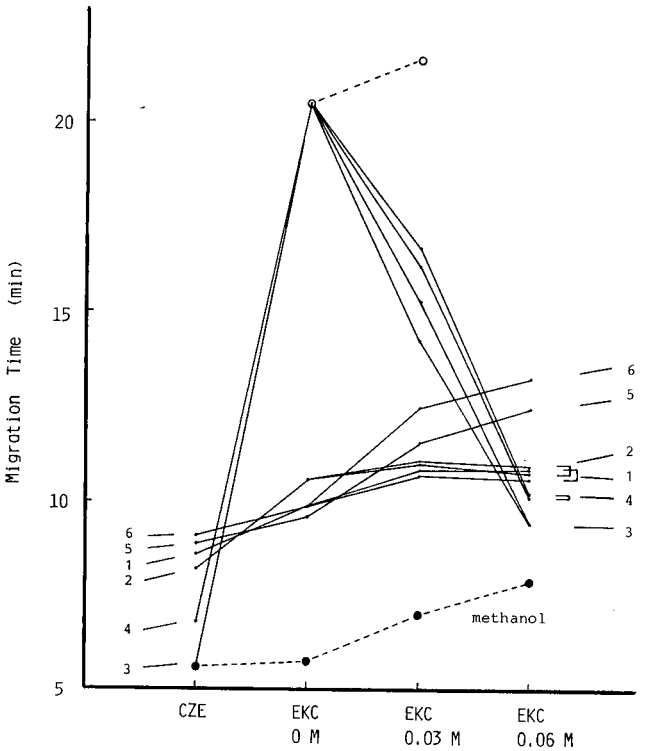


Fig. 4. Effects of γ-CD concentrations on the migration times and chiral recognition. Solute numbers as in Fig. 1. CZE, 0.02 *M* phosphate–borate buffer of pH 9.0. EKC, 0.05 *M* SDS added to CZE buffer. Open and closed circles represent the migration times of the micelle and methanol, respectively. Other conditions as in Fig. 2.

CD-MEKC and HPLC with CDs, where β-type CDs are the most effective [9]. The difference is interpreted in terms of the presence of the surfactant monomer in CD-MEKC.

Concentration effects of CDs on migration and chiral recognition

The effects of CD concentration on the migration times and chiral recognition were investigated using γ-CD over the concentration range 0–0.06 *M*. The results are shown in Fig. 4, with the results of CZE and MEKC without addition of CDs. The migration times or capacity factors of the solutes, especially of aromatic compounds except two achiral barbiturates, were considerably reduced with an increase in the CD concentration. It is therefore useful to use CDs in MEKC of lipophilic compounds, which migrate near the migration time of the micelle and cannot be resolved by the SDS solution alone, as reported previously [16,20].

In SDS solution containing CDs, a solute is distributed among three phases as mentioned above. A CD will not be solubilized by the micelle and migrate with the same velocity as that of the electroosmotic flow, except for methylation-type CDs. The stable inclusion-complex formation of the solute with the CD thus brings about a faster migration of the solute under the experimental conditions (pH 9.0), where the

electroosmotic flow is stronger than the electrophoretic mobility of the micelle [3].

As for the enantiomers, inclusion-complex formation with the CD can provide diastereomeric pairs of complexes. The difference in the stability of the diastereomeric inclusion complexes permits successful chiral separation. Theoretically, the optimum $\tilde{k}'$ for the highest resolution in MEKC can be calculated by the equation $\tilde{k}' = (t_{mc}/t_0)^{1/2}$, where t_{mc} and t_0 are the migration time of the micelle and that of an unincorporated solute, respectively [21]. The migration velocity of the solute in CD-MEKC should then be adjusted through the CD or micelle concentration to give the optimum resolution.

Effect of organic modifiers

Selectivity in MEKC can be manipulated through modification of micellar solution [1], in addition to varying the type of surfactant [22]. An organic modifier is useful for separations of aromatic amines [23] and cold medicine ingredients, in which ionic and non-ionic solutes are involved [24]. The effects of addition of methanol (10%) on the chiral recognition or capacity factors of four enantiomeric solutes were investigated using a 0.05 *M* SDS solution containing 30 m*M* γ-CD. The results are summarized in Table III. A typical chromatogram of the enantiomers of 2,2'-dihydroxy-1,1'-dinaphthyl and 2,2,2-trifluoro-1-(9-anthryl)ethanol is shown in Fig. 5. For these lipophilic compounds, the addition of an organic modifier was effective in changing the capacity factors and peak shapes. The capacity factors of the solutes were reduced by the addition of methanol, although their migration times increased because of the reduction of the electroosmotic flow. Chiral recognition of the enantiomers became poor and a single peak was observed from the enantiomers of pentobarbital. Methanol was probably included in the CD cavity, which will be unfavourable for the chiral recognition of the enantiomers of these barbiturates.

Effects of chiral additives

To enhance the chiral recognition of the solutes (from insufficient separation as shown in Fig. 2), the effect of some chiral additives, which can be included in the CD cavity, on the chiral recognition of the enantiomers was investigated. The effects of

TABLE III

EFFECTS OF METHANOL ADDITION ON SEPARATION FACTOR AND CAPACITY FACTOR

Buffer, 0.05 *M* SDS buffer solution of pH 9.0 containing 30 m*M* γ-CD.

Solute	No methanol		10% methanol	
	$\tilde{k}'$	α	$\tilde{k}'$	α
Thiopental	1.048	1.06	0.922	1.03
	1.114		0.950	
Pentobarbital	1.150	1.03	0.980	1
	1.194		–	
Dinaphthyl	3.055	1.34	2.486	1.27
	4.089		3.150	
Anthrylethanol	5.269	1.15	3.890	1.10
	6.055		4.297	

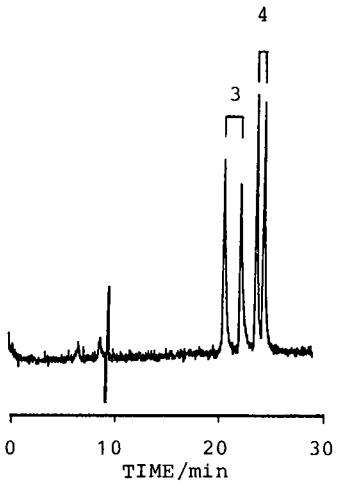


Fig. 5. Chiral separation of two lipophilic compounds by the addition of methanol. Buffer, 10% methanol in the same buffer solution as in Fig. 2. Other conditions as in Fig. 2.

addition of sodium *d*-camphor-10-sulphonate to the SDS solution containing γ-CD on the chiral recognition of four enantiomeric solutes are summarized in Table IV. A typical chromatogram is shown in Fig. 6. The separation factor and resolution of the enantiomers were improved with increasing concentration of the chiral additive, except for 2,2′-dihydroxy-1,1′-dinaphthyl, whose capacity factors are the largest among the four solutes. An increase in $\tilde{k}'$ will reduce the resolution because of deviation from the optimum capacity factor mentioned above.

The effects of *l*-menthoxyacetic acid (sodium salt form) on the chiral recognition of the same solutes are summarized in Table V. The resolution increased with

TABLE IV

EFFECT OF SODIUM *d*-CAMPHOR-10-SULPHONATE ON THE ENANTIOSELECTIVITY

Buffer, 0.05 *M* SDS containing 30 m*M* γ-CD and *d*-camphor-10-sulphonate.

Solute	*d*-Camphor-10-sulphonate								
	0 m*M*			20 m*M*			40 m*M*		
	$\tilde{k}'$	α	R_s	$\tilde{k}'$	α	R_s	$\tilde{k}'$	α	R_s
Thiopental	1.048	1.06	1.76	1.052	1.06	1.87	1.167	1.07	2.18
	1.114			1.117			1.247		
Pentobarbital	1.150	1.03	0.83	1.225	1.03	1.06	1.493	1.04	1.29
	1.194			1.265			1.549		
Anthrylethanol	3.055	1.34	3.08	3.442	1.20	4.22	4.362	1.22	4.40
	4.089			4.145			5.315		
Dinaphthyl	5.269	1.15	2.29	9.033	1.12	2.08	56.0	1.33	1.34
	6.055			10.07			74.3		

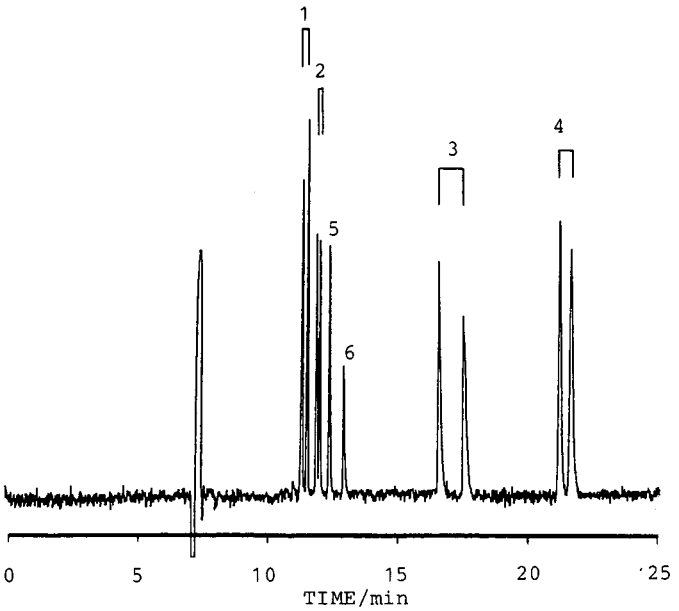


Fig. 6. Chiral separation by the addition of sodium *d*-camphor-10-sulphonate. Buffer, 20 m*M* sodium *d*-camphor-10-sulphonate in the same buffer solution as in Fig. 2. Other conditions as in Fig. 2.

the addition of the chiral compound up to 60 m*M*. In particular, the separation of two barbiturates and the peak shapes of the solutes were substantially improved. However, a high concentration of the chiral additive (90 m*M*) reduced resolution because of an increase in $\tilde{k}'$. A suitable concentration is selected to obtain the optimum resolution for the separation of each enantiomeric solute. A typical chroma-

TABLE V

EFFECT OF *l*-MENTHOXYACETIC ACID ON THE ENANTIOSELECTIVITY

Buffer, 0.05 *M* SDS containing 30 m*M* γ-CD and *l*-menthoxyacetic acid.

Solute	*l*-Menthoxyacetic acid								
	30 m*M*			60 m*M*			90 m*M*		
	$\tilde{k}'$	α	R_s	$\tilde{k}'$	α	R_s	$\tilde{k}'$	α	R_s
Thiopental	1.103	1.07	1.77	1.095	1.07	1.97	1.320	1.06	1.99
	1.178			1.167			1.398		
Pentobarbital	1.391	1.03	0.91	1.461	1.04	1.18	1.843	1.03	0.96
	1.436			1.515			1.903		
Anthrylethanol	9.751	1.49	3.21	11.99	1.27	3.66	17.27	1.24	3.14
	14.56			15.17			21.47		
Dinaphthyl	21.04	1.25	2.31	21.26	1.26	2.40	29.03	1.38	3.05
	26.40			26.70			40.20		

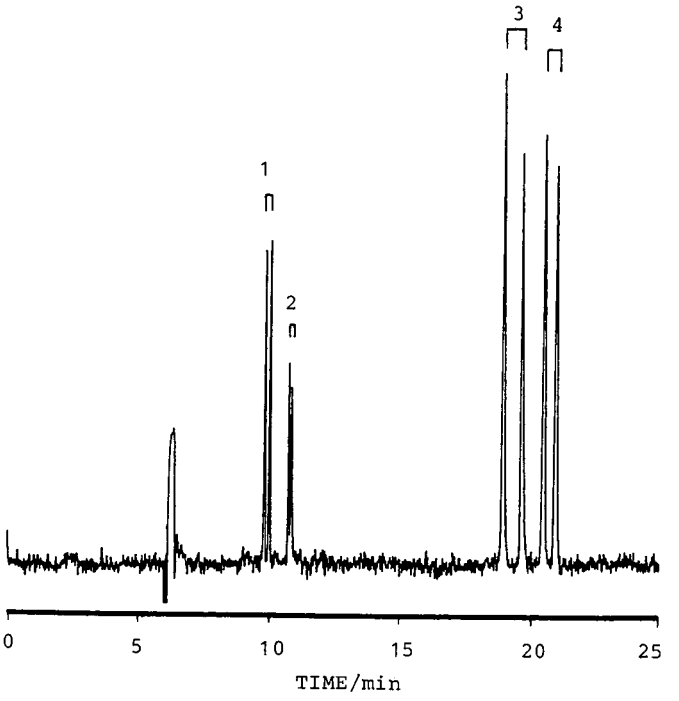


Fig. 7. Chiral separation by the addition of *l*-menthoxyacetic acid. Buffer, 0.06 *M* *l*-menthoxyacetic acid in the same buffer solution as in Fig. 2. Other conditions as in Fig. 2.

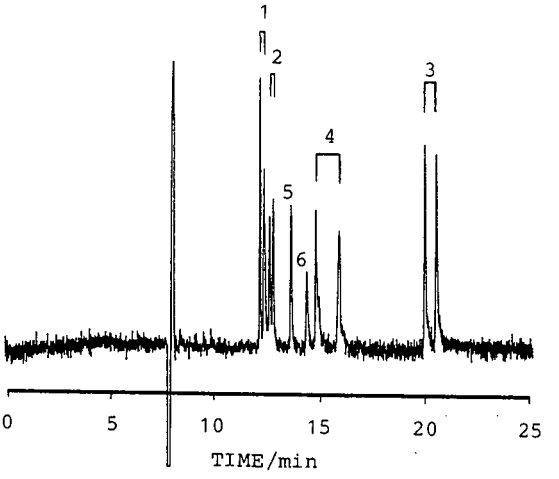


Fig. 8. Chromatogram of four enantiomeric solutes and two achiral solutes. Buffer, 0.05 *M* SDS solution of pH 9.0 containing 40 m*M* γ-CD and 20 m*M* sodium *d*-camphor-10-sulphonate. Other conditions as in Fig. 2.

togram obtained with addition of 60 m*M* *l*-menthoxyacetic acid is shown in Fig. 7. The migration times and capacity factors of the solutes increased with an increase in the concentration of these chiral additives, as shown in Tables IV and V.

This marked improvement in enantioselectivity may be ascribed to the inclusion of these chiral additives, which enhance the chirality of the CD cavity. The solute will be included in the γ-CD cavity together with the chiral additive. The cavity of γ-CD is large enough to include these two compounds in comparison with other CDs. It is well known that γ-CD often includes two molecules of the guest compound [19]. The interactions of the solute with both the CD and the chiral additive, through the hydrophobic or ionic portions, will probably give enhanced enantioselectivity.

Optimum chiral separation will be obtained by adjusting the concentrations of CDs and chiral additives. The addition of the former reduces the migration time of the solute; in contrast, the latter increases that of the solute. A chromatogram of four enantiomeric solutes and two achiral solutes is shown in Fig. 8, where a 0.05 *M* SDS solution of pH 9.0 containing 40 m*M* γ-CD and 20 m*M* sodium *d*-camphor-10-sulphonate was employed.

Chiral separation of aromatic compounds

The enantiomers of 2,2′-dihydroxy-1,1′-dinaphthyl were successfully resolved by using γ-CD and partially resolved by TM-β-CD. Chiral separation of 2,2,2-trifluoro-1-(9-anthryl)ethanol was only successfully achieved by using γ-CD. However, successful chiral separation of the enantiomers of 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate was achieved by using DM-β-CD and TM-β-CD rather than γ-CD. This result also differs from that of the chiral recognition of two barbiturates, as shown in Table II. This indicates that the ionic group of the solute affects the chiral recognition in CD-MEKC. The selection of the pH of the buffer solution is important for the

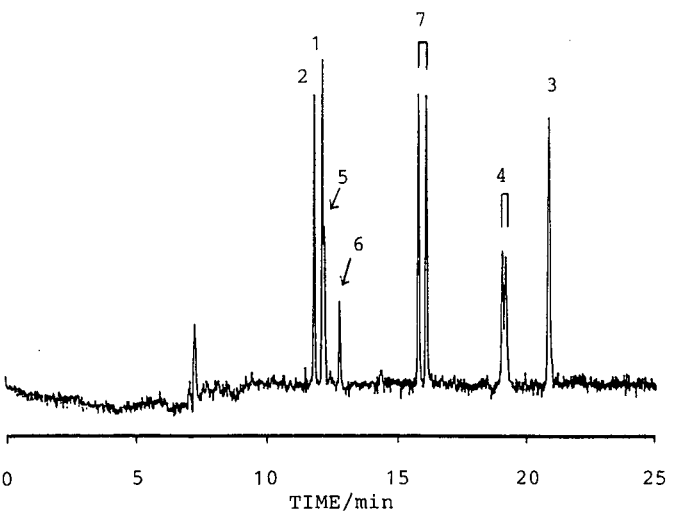


Fig. 9. Chiral separation using TM-β-CD. Buffer, 0.05 *M* SDS solution of pH 9.0 containing 15 m*M* TM-β-CD, 4 *M* urea and 40 m*M* sodium *d*-camphor-10-sulphonate. Other conditions as in Fig. 2.

successful chiral recognition of ionic enantiomeric solutes, in addition to the concentration and type of CDs. An example of the separation of 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate is shown in Fig. 9, where the enantioselectivity is enhanced by adding sodium *d*-camphor-10-sulfonate.

Chiral separation of barbiturates

The separation of four barbiturates including two enantiomeric drugs was investigated by using several modes of capillary electrophoresis such as CZE and MEKC. As for the chiral separation of the enantiomers, addition of γ-CD to the SDS

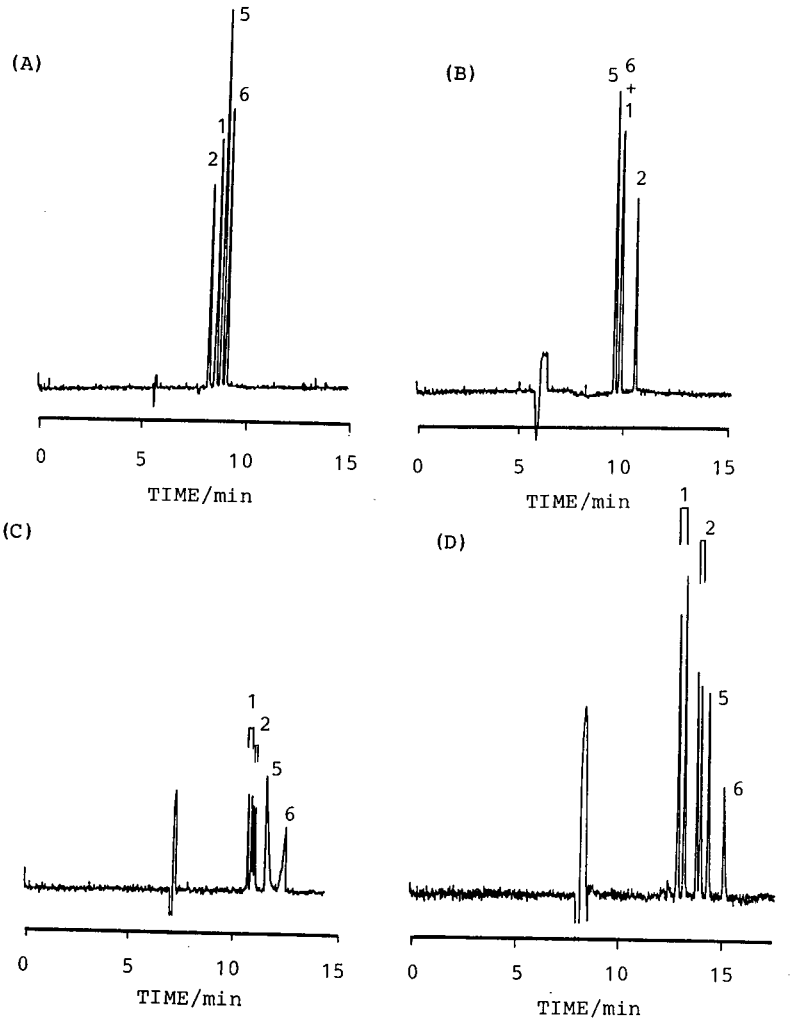


Fig. 10. Separation of four barbiturates including two enantiomeric drugs using different modes: (A) CZE using a 0.02 *M* phosphate–borate buffer solution of pH 9.0; (B) MEKC mode, 0.05 *M* SDS added to buffer in (A); (C) MEKC mode, 30 m*M* γ-CD added to buffer in (B); (D) CD-MEKC mode, 40 m*M* γ-CD and 40 m*M* sodium *d*-camphor-10-sulphonate added to buffer in (B). Other conditions as in Fig. 2.

solution was useful and a chiral additive in the SDS solution enhanced the enantioselectivity. These chromatograms are shown in Fig. 10. The migration times of thiopental and pentobarbital were reduced more than those of phenobarbital and sodium barbital by the γ-CD addition and therefore the migration order changed. The successful chiral separation of two enantiomeric drugs can be ascribed to the strong tendency of these drugs to interact with γ-CD. All the solutes including each enantiomer were separated by MEKC using γ-CD and sodium *d*-camphor-10-sulphonate as shown in Fig. 10D.

CONCLUSIONS

It was found that enantiomers of some drugs and chemicals can be successfully separated by CD-MEKC with SDS solutions. Chiral recognition was dependent on the type of CD and especially the cavity size. γ-CD was the most useful of five CDs studied and addition of some chiral additives such as sodium *d*-camphor-10-sulphonate or *l*-menthoxyacetic acid to the SDS solution containing CDs enhanced the enantioselectivity. Optimum capacity factors of the enantiomers for high resolution can be adjusted through the concentrations of CDs and chiral additives. Ionic interaction of the solute was one of the important facto ·s for the chiral recognition of ionic enantiomers.

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Non-equilibrium integral Doppler anemometric analysis of particle mixtures in a channel flow using an intrinsic hydrodynamic focusing force biased by another force

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ABSTRACT

Integral Doppler anemometric (IDA) analysis of particles exploits the strict correlation between the lateral position and the mean-time longitudinal velocity of a particle in a laminar flow. If the flow velocity profile is known, this correlation enables the transverse concentrational profile of a particle mixture to be measured by registering the Doppler frequency shifts for the particles simultaneously over the whole cross-section of a flow. IDA analysis allows one to detect the particle fractions in a channel flow with a transverse field applied [field-flow fractionation (FFF) arrangement] long before the longitudinal separation, and even before the complete transverse separation of fractions has occurred. This means a sharp decrease in the channel length and analysis time necessary compared with analytical FFF. The theory of IDA analysis is considered, then the intrinsic hydrodynamic lateral focusing force, naturally arising in a channel flow, is biased by another lateral (constant) force of any nature. Such a combination allows one to measure various physico-chemical parameters of particles working in the focusing regime favourable for registration. The theory is considered in a non-diffusive approximation for stationary, but laterally non-equilibrium, conditions. The theoretical relationships are given and the main characteristic features are considered for IDA analysis in a flat channel with Poiseuille or Couette flow for the three main ranges of external lateral force magnitude compared with the intrinsic hydrodynamic focusing force: (1) when two (different) lateral focusing positions still exist for particles; (2) when one lateral focus is left; and (3) when no lateral focusing occurs, and all the particles are drifting towards one wall. The transverse concentration profiles of particles and corresponding IDA spectra are calculated. The non-equilibrium IDA analytical separation of micrometre-size particles in a flow is demonstrated experimentally in the hydrodynamic focusing regime, using gravity as an external biasing force. The necessary channel length was *ca.* 10 cm, the analysis time *ca.* 30 s.

INTRODUCTION

The separation of particles in a flow and transverse external field [field-flow fractionation (FFF)] is a well established method for the analysis of particle mixtures [1,2]. The FFF process results in the formation of separated concentration zones along the channel, each zone corresponding to a certain fraction, which can be detected (or collected) at the outlet of a channel. For inherent physical reasons this process is completed only long after the establishment of a transverse separation of particles in a lateral field. As a consequence, it usually requires a 10–100-fold longer time than the

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process of particle transverse equilibration. For purely analytical purposes it is sufficient to register the transverse separation of particle fractions. Such a possibility is provided by integral Doppler anemometry (IDA) analysis, a recently developed technique for the rapid measurement of the transverse concentration profiles of particles in a laminar flow [3–5]. The basic idea of this method is that the flow velocity profile in a channel sets a definite relationship between the lateral position of a particle and its mean-time velocity along the flow. This local velocity can be measured via the Doppler frequency shift of the scattered light frequency, the corresponding local concentration of particles being measured by the scattered light intensity. Hence the integral Doppler spectrum of particles in a flow, measured from the whole cross-section of a channel, gives the transverse concentration profile of particles, provided that the flow velocity profile is known.

The IDA analysis of particle mixtures in a flow requires, similarly to analytical FFF [1,2], some intrinsic or externally applied force for transverse separation of particles in a channel. However, it differs from the original FFF principle in the role of the flow velocity gradient. In FFF this gradient is necessary for the longitudinal separation of fractions, which completes the whole separation process and precedes the registration procedure. In IDA analysis this gradient is necessary for Doppler registration purposes only, providing the coordinate-velocity sweep of the channel cross-section. Therefore, the registration can be done before the establishment of longitudinal separation of fractions. What is more, in IDA analysis it is not necessary to wait until the completion of the transverse separation of particles, as soon as transient (non-equilibrium) transverse concentration profiles of fractions are distinguished. These two features predetermine the essentially shorter analysis times and channel lengths that can be achieved with the IDA analysis approach compared with analogous FFF analytical schemes. At the same time, the IDA technique requires an optically transparent section of a channel wall, which may present additional difficulties in practical design for some kinds of transverse field. From this point of view it seems very advantageous to use the intrinsic hydrodynamic focusing force, which allows the IDA analytical separation of particles in a flow to be accomplished without application of any external force [6].

The IDA analysis can be implemented in two versions: (a) the non-stationary scheme with instantaneous local probe injection, as in FFF techniques; and (b) the stationary scheme with a time-constant homogeneous concentration distribution of the particle mixture at the channel entrance. The latter version is more suitable for experimental realization, so it was chosen for this work. In the stationary version the measuring channel with the lateral force applied is included in a closed circuit containing the suspension being analysed. This suspension circulates with a constant velocity, and is stirred homogeneously outside the channel. Thus, the stationary concentration distributions of particles in a channel flow are established due to the action of the lateral force and velocity gradient, being specific for each particle fraction. Near the channel entrance these distributions are essentially non-equilibrium relative to the lateral field, while further along the channel they can transform into equilibrium ones, depending on the interrelation between the channel length, flow velocity and force magnitude.

The theoretical description of IDA particle analysis naturally splits into two independent tasks: (a) the calculation of the particle concentration distribution all over

the channel flow for the given temporal and boundary conditions; and (b) the calculation of the corresponding IDA spectra of suspension flow, taking into account the flow velocity profile, measuring geometry, illumination distribution and particle polydispersity. The final expressions for the shape of IDA spectra should contain certain adjustable parameters characteristic of particle fractions, which enable the analytical procedure to be accomplished.

This paper, like the previous one [6], considers IDA particle analysis under non-equilibrium field-flow conditions, *i.e.*, in the process of transverse equilibration of particles in a channel. The non-equilibrium scheme of IDA analysis seems the most advantageous over analogous FFF analytical schemes as far as analysis speed and channel length are concerned. The general theory was developed [6] using a kinematic (non-diffusive) approximation. This approximation is justified for the case of sufficiently large particles (>1 μm size) in the process of transverse equilibration, because diffusion plays a minor role in the transformation of the particle concentration distribution in that case. In this paper we consider two schemes of stationary non-equilibrium IDA particle analysis in a flow which are of most interest for practical applications: (a) with a constant lateral force of any nature; and (b) with the intrinsic hydrodynamic focusing force, biased by a constant lateral force. Analytical separation experiments for the latter case are also presented.

THEORETICAL

Consider a laminar dilute suspension flow in a flat channel of width $2h$. We shall use the dimensionless coordinate system in units of h with the z-axis along the flow, x-axis perpendicular to the channel walls and the origin at the middle of channel inlet. Let $v_z(x) = v_{\|}u(x)$ be the flow velocity profile and $F_x(x) = F_0\varphi(x)$ be the profile of lateral force acting on particles suspended in a flow. Here $v_{\|} > 0$, $F_0 = \pm|F_0|$ are characteristic values and $u(x)$, $\varphi(x) > 0$ are dimensionless profiles. Let μ be a characteristic parameter of particle species and $C_0(\mu,x_0)$ be the corresponding stationary concentration distribution at the channel inlet. Then, in a non-diffusive approximation, the stationary concentration distribution $C(x,z)$ of particles in a channel flow is given by [6]

$$C(x,z) = \int C_0\{\mu,x_0(x,z,\mu)\}C(x,z,\mu)\,\mathrm{d}\mu \tag{1a}$$

$$C(x,z,\mu) = \frac{\varphi\{x_0(x,z,\mu)\}}{\varphi(x)} \tag{1b}$$

$$z = \mu\int_{x_0}^{x}\frac{u(x)}{\varphi(x)}\mathrm{d}x, \qquad \mu = \frac{6\pi\eta a v_{\|}}{F_0} \equiv \frac{v_{\|}}{v_{\perp}} \tag{1c}$$

where a is the particle radius, η is the fluid viscosity and $v_{\perp}$ is the characteristic lateral drift velocity of a particle. Eqn. 1c is the trajectory equation, which connects the actual particle's lateral coordinate x with its starting coordinate x_0 and *vice versa*. Particles

are assumed to drift according to Stokes law, and to have the unperturbed local flow velocity in the z direction.

In the following discussion we adopt two restrictions. First, we assume homogeneous concentrational profiles at the inlet of a channel, *i.e.*, $C_0(\mu,x_0)$ being independent of x_0. Second, we consider the particle mixture with a discrete set of fractions, which in turn can be well exemplified by a binary mixture owing to additivity of results:

$$C_0(\mu) = \frac{1}{\sqrt{2\pi}} \cdot \left\{ \frac{C_1}{\sigma_1} \cdot \exp\left[-\frac{(\mu - \mu_1)^2}{2\sigma_1} \right] + \frac{C_2}{\sigma_2} \cdot \exp\left[-\frac{(\mu - \mu_2)^2}{2\sigma_2} \right] \right\} \tag{2}$$

Here we assume a Gaussian-type parameter distribution of fractions near mean values μ_1, μ_2, with partial concentrations C_1, C_2 and widths σ_1, σ_2. In such a case the shape of the integral Doppler spectrum $S(\omega,z)$ of suspension flow, registered at some distance z from the channel inlet, is given approximately by [3,6]

$$S(\omega,z) \sim \int a^3 \psi(qa) C_0(\mu) \int_{-1}^{1} \frac{\varphi\{x_0(x,z,\mu)\}}{\varphi(x)} \delta[\omega - q v_{\parallel} u(x)] \mathrm{d}x \mathrm{d}\mu \tag{3}$$

where $\omega = 2\pi f$ is the angular frequency, q and $\psi(qa)$ are the light-scattering vector and indicatrix, respectively, and $\delta[y(x)]$ is the delta-function of $y(x)$.

Eqns. 1 and 3 give the necessary relationships between particle parameters a and μ and the shape of appropriate IDA spectra. They should be further specified for given profiles of lateral force and flow velocities.

Homogeneous lateral force, $\varphi(x) = 1$

This case is of special interest, being both practically important and easily tractable. To avoid unnecessary complications, let us assume that μ has the same sign for all particle fractions. In that case, owing to the action of lateral force, all the particles are gradually displaced to the channel wall lying at $x = \mathrm{sign}(\mu)$. This means that for each particle fraction there exists the characteristic boundary trajectory $x = x_m(z,\mu)$. It starts from the point $x_0 = -\mathrm{sign}(\mu)$, $z_0 = 0$ and defines the position along the channel of a sharp edge of concentration distribution of this fraction: $C(x,z) \equiv 0$ (in a non-diffusive approximation!) for $x < x_m(z,\mu)$ if $\mu > 0$, or for $x > x_m(z,\mu)$ if $\mu < 0$. This trajectory's equation is obtained by substitution of $x_0 = -\mathrm{sign}(\mu)$ into eqn. 1c and has the following forms for plane Poiseuille and Couette flows, respectively:

$$u(x) = 1 - x^2: \qquad z = \frac{1}{3} \cdot |\mu| \cdot [2 + (3x_m - x_m^3)\mathrm{sign}(\mu)] \tag{4a}$$

$$u(x) = \frac{1}{2}(1 + x): \qquad z = \frac{1}{4} \cdot |\mu| \cdot [2 + (2x_m + x_m^2 - 1)\mathrm{sign}(\mu)] \tag{4b}$$

The shapes of these boundary trajectories are shown in Fig. 1a for two values of $|\mu|$. Note that the trajectories are strongly dependent on $|\mu|$. In the case of Couette flow they also depend on the sign of μ (direction of the lateral force) owing to the lack of central-plane symmetry.

The final point, $x_m = \text{sign}(\mu)$, $z = z_f$, of the boundary trajectory defines the effective distance from the channel inlet corresponding to the particles quasi-equilibration in the lateral field. From eqns. 4 we obtain the general expressions for z_f in the case of Poiseuille (P) and Couette (C) flows, and also equations specified for sedimentation–flotation non-equilibrium FFF (NEFFF), tested experimentally in this work:

$$u(x) = 1 - x^2: \quad (z_f)_P = \frac{4}{3}|\mu| = \frac{8\pi\eta a v_{||}}{|F_0|}; \quad (z_f)_P = \frac{6\eta v_{||}}{a^2 g|\rho_1 - \rho_0|} \tag{5a}$$

$$u(x) = \frac{1}{2}(1 + x): \quad (z_f)_C = \frac{3}{4}(z_f)_P = |\mu| \tag{5b}$$

The shape of the transverse concentration profile which enters the expression for the IDA spectrum (eqn. 3) is determined by eqn. 1a. It shows that for $\varphi(x) =$ constant and a strictly monodisperse fraction this profile has (in a non-diffusive approximation!) a step-like shape at any $z < z_f$ and regardless of the flow velocity profile. The edge position of this step is determined by the boundary trajectory $x = x_m(z,\mu)$, described by eqns. 4. This is accompanied by (mathematical) singularity in $C(x,z)$ at the opposite wall of a channel. In reality, such singularity and the sharp edge at $x = x_m(z,\mu)$ are smeared more the greater is the distance z from the channel inlet, owing to particle

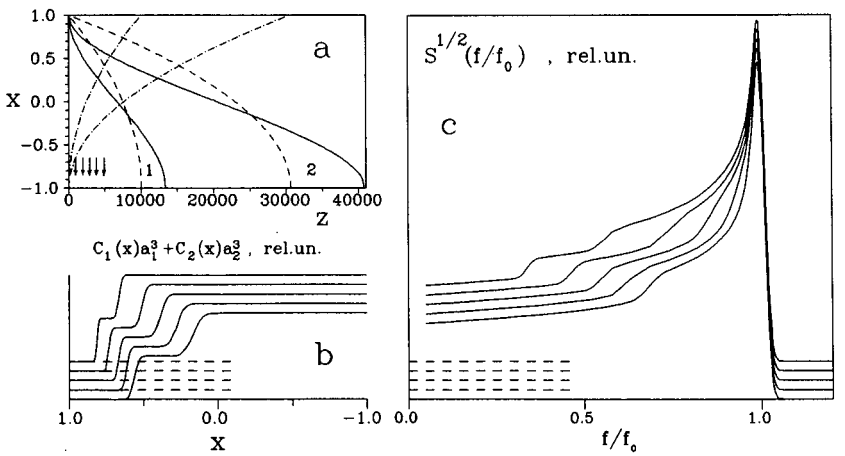

Fig. 1. (a) Boundary trajectories for a binary mixture of particles in a channel flow with a constant lateral force, calculated for plane Poiseuille (———) and Couette flows. – – –, The force is directed to the stationary channel wall; –·–·–, to the moving wall. Fractionation parameter: (1) $\mu_1 = -1 \cdot 10^4$; (2) $\mu_2 = -3.1 \cdot 10^4$. (b) Transverse profiles of a stationary volumetric concentration of a binary particle mixture in a plane Poiseuille flow, calculated for several distances z along the channel [marked by arrows in (a)]. Curves are shifted vertically for clearness. $(\sigma_1/|\mu_1|) = (\sigma_2/|\mu_2|) = 0.05$; $C_1 a_1^3 = C_2 a_2^3$. (c) Integral Doppler spectra of suspension flow, corresponding to the concentrational profiles in (b). $C_1 = 0.16$, $C_2 = 0.84$.

diffusion and polydispersity. Whereas the diffusion can be reasonably neglected in the case of sufficiently large particles, the degree of polydispersity does not correlate *a priori* with the size (or some other parameters) of the particles. Therefore, in view of the strong μ dependence of the boundary trajectories (see Fig. 1a and eqns. 4), the inherent polydispersity of any real particle system should necessarily be taken into account in the theory. This necessity, in turn, additionally justifies the use and extends the application range of the non-diffusive approximation adopted here.

Fig. 1b and c show transverse concentration profiles and corresponding IDA spectra for a binary mixture of particles (eqn. 2) in a Poiseuille flow, calculated according to eqns. 1–3. For a negligibly small instrumental broadening of a Doppler line, the relationships between the IDA spectrum and the concentration profile are as follows:

Poiseuille flow, $u(x) = 1 - x^2$:

$$S(\omega,z) \sim \frac{1}{\sqrt{1-\dfrac{\omega}{\omega_0}}} \int a^3 \psi(qa) C_0(\mu) \left[C\left\{ \sqrt{1-\frac{\omega}{\omega_0}}, z, \mu \right\} + C\left\{ -\sqrt{1-\frac{\omega}{\omega_0}}, z, \mu \right\} \right] \mathrm{d}\mu \quad (6a)$$

Couette flow, $u(x) = \frac{1}{2}(1 + x)$:

$$S(\omega,z) \sim \int a^3 \psi(qa) C_0(\mu) C\left\{ \left(2\frac{\omega}{\omega_0} - 1 \right), z, \mu \right\} \mathrm{d}\mu \quad (6b)$$

Here $\omega_0 = qv_{\|}$. Eqns. 6 show that [in view of the weak $\psi(qa)$ dependence on a] the shape of the IDA spectrum is related to the profile of the volumetric concentration of the particles, a^3C. This relationship reduces to simple changes of variables, specified by the corresponding spatial dependence of flow velocity. The central-plane symmetry of Poiseuille flow leads to additional summation of concentration profiles over "positive" ($0 \leqslant x \leqslant 1$) and "negative" ($-1 \leqslant x \leqslant 0$) halves of a channel. This feature should be taken into account in the course of $C(x,z)$ reconstruction from the measured spectra, because $C(x,z)$ has no central-plane symmetry. In the case of Couette flow the relationship mentioned is especially simple. Here the shape of the IDA spectrum replicates the shape of $C(x,z)$ owing to the linear connection between the Doppler frequency and the lateral coordinate of a particle, which is especially convenient for the measurements.

Hence Fig. 1 illustrates situations for both Poiseuille and Couette flows. Together with eqns. 1 and 4, it shows that for the discrete set of fractions in a particle mixture in a channel flow with a constant lateral force, the stationary non-equilibrium transverse concentration profiles and the corresponding IDA spectra have characteristic shoulders corresponding to each fraction. This gives the possibility of analytical fractionation of a mixture relative to the parameter μ, which contains the size and some other relevant parameter of a particle (see eqns. 1 and 5). The corresponding values of μ can be found from the measured shoulders' positions $x_m(z,\mu)$ and the known registration position z, using eqns. 4 and, for a more precise evaluation, eqns. 1. Fig. 1a

and eqns. 4 enable one to optimize the separation conditions by choosing the appropriate z and $v_{\|}$. The use of Couette flow in IDA particle analysis with a constant lateral force is obviously preferable to Poiseuille flow owing to the much simpler data processing.

Combination of intrinsic hydrodynamic and constant external force

A particle in a channel flow undergoes the action of intrinsic focusing hydrodynamic force, acting in a lateral direction [5–10]. In the case of Poiseuille flow there are two symmetrical non-central focusing positions, $\pm x_f$, the exact value $0 < |x_f| < 1$ being dependent on the ratio a/h [6,8,9], whereas in Couette flow the focusing position is the central plane of a channel [10]. The characteristic magnitude of this focusing force depends strongly on a/h, giving the possibility of using this force for the analytical fractionation of particles in a flow [6]. The combination of this force with the second (external) lateral force of any physical nature opens up a very promising possibility of analytical fractionation of particles relative to various parameters, using an efficient and precise focusing regime of IDA registration.

We consider below the more complicated case of Poiseuille flow, with the obvious extension to Couette flow. In the former case, as was pointed out previously [6], the intrinsic hydrodynamic force can be well approximated by the equation

$$F_x = F_0\varphi(x);\ F_0 = \frac{9\pi}{4}v_{\|}^2 \cdot \frac{\rho_0 a^2}{1 - x_f^2}\left(\frac{a}{h}\right)^2;\ \varphi(x) = x(x_f^2 - x^2) \tag{7}$$

Combining eqn. 7 with some constant external force F_e, we obtain from eqn. 1 the following trajectory equation:

$$z = \mu\int_{x_0}^{x}\frac{1 - x^2}{x(x_f^2 - x^2) + \lambda}\,dx \tag{8}$$

where $\lambda = (F_e/F_0)$. Hence, the particle fraction is now characterized by two parameters, μ and λ. The first contains the size of a particle (eqn. 7), and the second contains the combination of size and some other particle parameter, which is relevant for the external field. Integrating eqn. 8, we obtain

$$\frac{z}{\mu} = A\ln\left(\frac{x - x_1}{x_0 - x_1}\right) + B\ln\left(\frac{x - x_2}{x_0 - x_2}\right) + C\ln\left(\frac{x - x_3}{x_0 - x_3}\right) \tag{9}$$

where

$$x_1 = \frac{2}{\sqrt{3}}x_f\cos\left[\frac{1}{3}\arcsin\left(\frac{\lambda}{\beta}\right) - \frac{5\pi}{6}\right]$$

$$x_2 = \frac{2}{\sqrt{3}}x_f\cos\left[\frac{1}{3}\arcsin\left(\frac{\lambda}{\beta}\right) + \frac{\pi}{2}\right]$$

$$x_3 = \frac{2}{\sqrt{3}} x_f \cos\left[\frac{1}{3}\arcsin\left(\frac{\lambda}{\beta}\right) - \frac{\pi}{6}\right]$$

$$\beta^{-1} = \frac{3\sqrt{3}}{2x_f^3} \qquad x_f > 0$$

$$A = (x_1^2 - 1)(x_1 - x_2)^{-1}(x_1 - x_3)^{-1}$$

$$B = (1 - x_2^2)(x_1 - x_2)^{-1}(x_2 - x_3)^{-1}$$

$$C = (x_3^2 - 1)(x_1 - x_3)^{-1}(x_2 - x_3)^{-1}$$

Here for $\lambda > \beta$ the roots x_1, x_2 and x_3 are complex, but the whole expression for z always remains real. Eqns. 8 and 9 show that, depending on the relative magnitude λ of the external force, three qualitatively different focusing regimes are possible: (1) if $0 \leqslant |\lambda| < \beta$, then there are two lateral focusing positions, x_1 and x_3, which are the initial (at $F_e = 0$) focusing positions, shifted from $\pm x_f$ owing to external force; (2) if $\beta < |\lambda| < (1 - x_f^2)$, then only one focusing position is left, *e.g.*, x_3 for $\lambda > 0$; (3) if $(1 - x_f^2) < |\lambda|$, then the external force is dominating, and the particles are drifting to the appropriate wall. The latter regime is similar to the case of a constant lateral force, considered above.

All three focusing regimes are illustrated by Figs. 2 and 3, where the corresponding boundary trajectories (eqn. 9, $x_0 = 1$) and transverse concentrational profiles (eqns. 1 and 9) are plotted for a single fraction of particles (eqn. 2 with a single term). Note that the concentration profiles are plotted as the sums of profiles, computed for $0 \leqslant x \leqslant 1$ and $-1 \leqslant x \leqslant 0$. This is done according to the form in which they enter the IDA spectrum of Poiseuille suspension flow (eqn. 6a), and in which they are evaluated from the measured spectra. As Fig. 3 shows, in the first regime a transverse concentration profile of each fraction has two characteristic peaks, in the second regime a peak and a shoulder and in the third regime only a shoulder. The

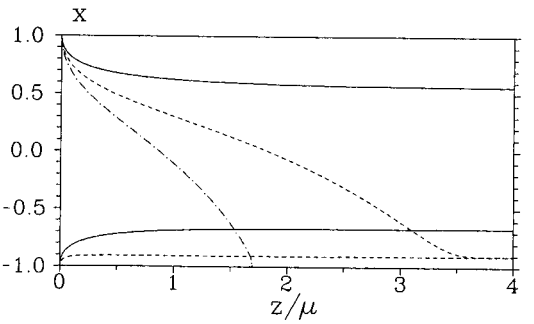

Fig. 2. Boundary trajectories for a single fraction of particles in a plane Poiseuille flow, calculated for three characteristic examples of the ratio between the constant external and intrinsic hydrodynamic lateral forces: ———, $0 < |\lambda| < \beta$; – – –, $\beta < |\lambda| < (1 - x_f^2)$; —·—·—, $-|\lambda| > (1 - x_f^2)$. $x_f = 0.62$; $\lambda = 0.04$, 0.4 and 0.8, respectively. These trajectories give the positions of concentration peaks and shoulders.

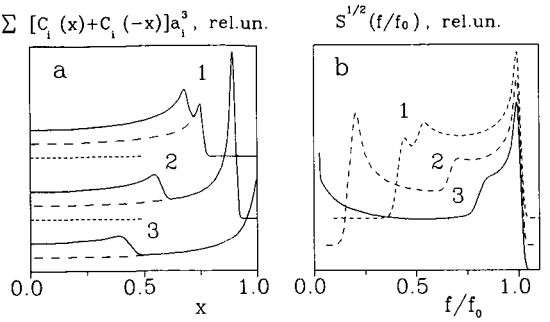

Fig. 3. (a) Transverse profiles of a stationary volumetric concentration of a single fraction of particles (summed over two halves of a channel), calculated for a plane Poiseuille flow for the three main regimes of combined action of a constant external and intrinsic hydrodynamic lateral forces. $\mu = -1.24 \cdot 10^4$; $(\sigma/|\mu|) = 0.07$; $z = 4 \cdot 10^3$; $x_f = 0.62$. (1) $\lambda = 0.086$; (2) $\lambda = 0.38$; (3) $\lambda = 0.76$. (b) Integral Doppler spectra of suspension flow, corresponding to the concentration profiles in (a).

positions of these features are determined by the boundary trajectories (eqn. 9 and Fig. 2) corresponding to the mean μ and λ values in the appropriate eqn. 2, written for a single fraction.

The case of Couette flow, where the intrinsic hydrodynamic force has only one (central-plane) focusing position [10], corresponds qualitatively to the second and third focusing regimes of Poiseuille flow.

Fig. 3b shows the IDA spectra of suspension flow calculated for the concentration profiles in Fig. 3a. They evidently retain all characteristic peaks and shoulders mentioned for concentration profiles. That makes unnecessary the procedure of complete reconstruction of $C(x,z)$ from $S(\omega,z)$ in order to evaluate the μ and λ parameters. This can be done by measuring the spectral positions of peaks and shoulders only, with the subsequent scaling according to $x = \sqrt{1 - (\omega/\omega_0)}$.

As eqn. 9 shows, the evaluation of μ and λ can be done in two ways. If the x_f value is known beforehand, then it is sufficient to measure the positions of two peaks (or peak and shoulder) in the presence of an external force. However, the experiments showed that, contrary to theoretical predictions [10], the position of x_f for very narrow channels depends on the ratio of particle size to channel width (a/h) [6,8,9]. In such a case the additional measurements of a concentration peak's position are necessary under the same flow conditions, but in the absence of external force. That gives three sets of (x_m,z) values for determining of x_f, μ and λ from eqn. 9.

EXPERIMENTAL

For the present experiments the simplest possible arrangement was used, with gravity as an additional external force. For this purpose the flat glass channel (y–z plane) was placed horizontally, whereas in the previous experiments [4–6] its central plane was vertical, the z-axis being horizontal. The laser beams and the scattered light were rotated by 90° before and after the channel, respectively, in order not to change the remainder of the optical and registration set-up [4,5]. The channel width $2h$ was 180 μm, channel length 250 mm and the third dimension was 8 mm. The flow velocity at the

axis was about 1 cm/s. The measured samples were dilute suspensions (10^5–10^7 particles/cm^3) of human erythrocytes ($a_1 \approx 3{,}5$ μm) and latex particles ($a_2 = 2$ μm) in a standard phosphate-buffered saline at pH 7.3–7.5.

Fig. 4a shows the measured IDA spectra of a pure latex suspension, a pure erythrocyte suspension and their mixture. Fig. 4b shows the volumetric concentrational profiles (summed over upper and lower parts of a channel), reconstructed from these spectra using eqn. 6a. The spectra and profiles of pure suspensions have two characteristic features, namely the peak and the shoulder, which shift systematically with a change of either registration point along the channel or flow velocity. The spectra, measured for mixtures, seem to be the superpositions of spectra measured for pure components (Fig. 4), the heights of peaks and shoulders following the corresponding concentrations of species in a mixture.

DISCUSSION AND CONCLUSIONS

Comparison of volumetric concentration profiles, reconstructed from the measured IDA spectra (Fig. 4), with the theoretical curves (Figs. 2 and 3) shows that the measurements were done in the second focusing regime. In this regime lateral focusing occurs in the nearest vicinity of the lower channel wall only, giving a pronounced narrow peak in the IDA spectrum. In the upper half of a channel the sedimentation force outweighs the focusing hydrodynamic force, and the particles gradually sink to the lower focusing plane. The latter process is analogous to the case of a constant lateral force (eqns. 4 and 5 and Fig. 1), and gives a shoulder in the concentration profile and IDA spectrum (Fig. 4). This shoulder is more distinct and steep for erythrocytes (at $x \approx 0.42$) than for latex (at $x \approx 0.69$), which gives evidence for the higher degree of polydispersity of the latex suspension. In the case of the pure latex suspension, apart from the main shoulder (at $x \approx 0.69$), there is the smaller second shoulder at $x \approx 0.4$. This corresponds to the pairs of latex particles which were

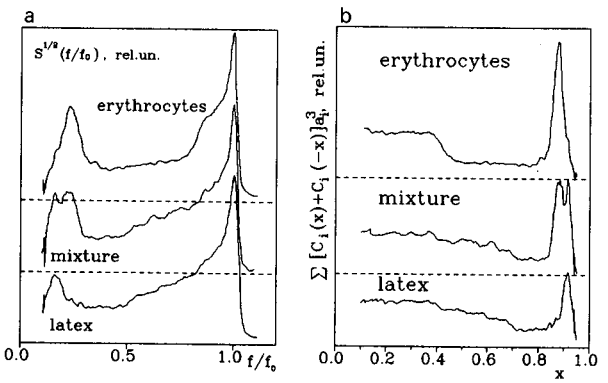

Fig. 4. (a) Integral Doppler spectra of channel flows of suspensions of erythrocytes and latex, and of their mixture ($C_1/C_2 \approx 0.15$), measured at $z = 2 \cdot 10^3$ ($zh = 180$ mm) and $v_{\|} = 0.86$ cm/s. (b) Transverse volumetric concentration profiles of particles in suspension flows, reconstructed from the corresponding IDA spectra. The formation of concentration peaks is due to the focusing hydrodynamic force, whereas their shift is due to gravity.

also detected in a small amount in the suspension on microscopic examination. With the mixtures the peaks and shoulders corresponding to the individual components can be distinctly resolved and correspond in position to those in the profiles of the pure suspensions. The values of size and material density of fractions evaluated from the measured IDA spectra using eqns. 7–9 agree with their known values.

Hence the experimental data show that, under the measuring conditions, the lateral focusing and the lateral separation of different particle fractions occur in a channel flow owing to the combined action of the intrinsic focusing hydrodynamic force and gravity. The separation occurs under laterally non-equilibrium conditions, which is evidenced by the presence of shoulders along with the peaks in the concentration profiles. These experiments demonstrate the possibility of non-equilibrium sedimentation–flotation IDA analysis of particle mixtures. The experimental set-up is characterized by an extremely simple channel design and a very short analysis time; the registration can be started 1 min after switching on the circulation of suspension, and takes *ca.* 30 s. The analysis can be done either in a constant force regime or in a focusing force regime. As Figs. 1, 3 and 4 show, the latter gives a better resolution of fractions owing to the formation of very narrow concentration peaks. The positions of these peaks in units of channel half-width h can be measured from IDA spectra with great accuracy and reproducibility, characteristic of laser Doppler anemometry [11], so the absolute error depends mainly on the absolute evaluation of $2h$. The evaluation of characteristic parameters of fractions can be done from the measured positions of peaks and shoulders, using the boundary trajectory diagrams in Figs. 1 and 2 and eqns. 4, 5 and 9. These diagrams can also be used for optimization of IDA analysis. The key optimization parameters are the coordinate z_R of IDA spectrum registration and the separation parameter of fraction μ, defined by eqns. 1 (see also eqns. 5). As Figs. 1 and 2 show, by varying z_R it is possible to obtain a better resolution of fractions in the preselected range of μ values, whereas by varying the flow velocity $v_{||}$ and fluid viscosity η which enter the μ expressions it is possible to shift the entire set of μ values of the mixture being analysed. The parameter μ also defines the effective distance along the channel, which corresponds to equilibration of the particle fraction in a constant lateral field (eqns. 5). As Fig. 1 shows, the optimum range of IDA registration positions z_R (*i.e.*, the necessary channel length) is less than *ca.* $0.5\ \mu_{min}\ h$, where μ_{min} is the minimum μ value for a mixture of fractions. This applies also to the externally biased hydrodynamic focusing force (eqns. 7–9). In the present experiments with gravity as a biasing lateral force, the necessary channel length was *ca.* 10 cm. Using centrifugation instead of gravity (although it requires a special optical design), it is possible to work with channels several centimetres long (see eqns. 5), the analysis time being several tens of seconds. For comparison, in high-speed versions of FFF analysis (thermal and steric) the analysis time is *ca.* 5 min and in sedimentation FFF it is *ca.* 30 min, typical channel lengths being several tens of centimetres [2]. It is interesting also to compare our experiments with the original sedimentation–flotation focusing field-flow fractionation experiments [12,13]. These experiments were done in a standard FFF regime, *i.e.*, under laterally equilibrium conditions. First, to create the focusing conditions and to obtain an efficient longitudinal separation of particles at the end of a channel, a specially designed density gradient of the carrier fluid and a modulated cross-section of a channel should be used [12,13]. This is not necessary in a non-equilibrium registration regime with the use of intrinsic hydrodynamic force.

Second, in the non-equilibrium scheme the registration position is obviously closer to the channel entrance, so the necessary channel length is considerably lower.

The experimental results obtained here and the theory developed demonstrate two possible general schemes of non-equilibrium IDA particle analysis: (1) using an externally applied lateral force, similar to conventional FFF schemes [1,2]; (2) using combinations of external fields of various natures with the intrinsic hydrodynamic focusing force. The second scheme seems very promising, because it gives the possibility of measuring various physico-chemical parameters of particles in a favourable focusing regime of IDA registration. For practical design of experimental set-ups it is essential that the intrinsic hydrodynamic force, and gravity, cannot be simply "switched" on and off. However, the former is strongly dependent on the $v_{\|}$ and a/h (eqn. 7), so it can be made negligibly small by using an appropriately wide channel and a sufficiently low flow velocity. The action of gravity can be either included in calculations or compensated for by matching the carrier fluid density ρ_0 or eliminated by using perpendicular forces geometry.

The theory of IDA analysis, developed in a non-diffusive approximation, proved to be efficient for (a) general qualitative analysis of a separation process in various experimental set-ups, with different kinds of lateral forces and flow velocities; (b) optimization purposes; and (c) quantitative evaluation of experimental data (for micrometre-size particles and, in some instances, for submicrometre-size particles). The use of a convective diffusion equation in the theory is not so important in the wide field of applications of non-equilibrium IDA analysis. The main reasons are first that the early stages of lateral equilibration of particles usually need to be considered, and second that the polydispersity of any real particle system in many instances plays the major role compared with diffusion in the smearing of concentration profiles in these stages.

In conclusion, it is necessary to point out that the method of IDA analysis does not involve the use of a laser Doppler anemometer as just another particle detector in FFF. It is based on another principle of analytical fractionation of particles under field-flow conditions, which does not require the preliminary longitudinal separation of fractions and, what is more, allows one to do the analysis even before the completion of the transverse separation of fractions. These features give major advantages in terms of analysis duration and necessary channel length. IDA analysis also gives the possibility, which is unattainable in conventional FFF, of performing mixture analyses in a continuous injection regime, when no longitudinal separation of fractions occurs in a channel. This is a very promising possibility, *e.g.*, for on-line product monitoring (indicative FFFF [6]). As for the choice of the detector in IDA, it is dictated primarily by the necessity to register the spatial dependence of particle velocity in a flow, which is the basic idea of the IDA method. In principle, this can also be done using some other physical phenomena, *e.g.*, by ultrasound scattering. However, at present such detectors are not at hand for this particular application, so the laser Doppler anemometer remains the only usable detector for IDA particle analysis. On the other hand, the laser Doppler anemometer can also be used for registration of particle fractions in conventional FFF schemes, where these fractions are separated longitudinally. However, owing to the non-stationary time conditions in FFF, such applications have specific features which are beyond the scope of the stationary analysis presented in this paper, and will be the subject of a future publication.

SYMBOLS

a	radius of a particle
$C(x,z)$	concentration of particle mixture in a flow
$C(x,z,\mu)$	concentration of particle fraction in a flow
$C_0(\mu)$	stationary homogeneous concentration at the channel entrance
C_1, C_2	relative concentrations of fractions in a binary mixture
$F_x(x)$ and F_0	lateral force and its magnitude
F_e	external biasing lateral force
f	frequency in Hz
g	acceleration due to gravity (or to centrifugal force)
h	half-width of a flat channel
q	laser light scattering wavenumber
$S(\omega,z)$	IDA power spectrum at a distance z from the entrance
$u(x)$	dimensionless velocity profile of a channel flow
$v_{\|\|}$	maximum flow velocity
x	lateral coordinate of a particle (in units of h)
x_0	lateral coordinate of a particle at the channel entrance
x_f	lateral position of the focusing point
$x_m(z,\mu)$	boundary trajectory of a particle fraction
z	particle coordinate along the flow (in units of h)
z_f	effective distance, corresponding to lateral equilibration
z_R	position of registration of IDA spectrum
β	$2x_f^3/3\sqrt{3}$
η	fluid viscosity
$\lambda = (F_e/F_0)$	characteristic ratio between external biasing and intrinsic hydrodynamic forces
μ	characteristic separation parameter of a particle fraction
ρ_0	fluid density
ρ_1	particle material density
σ	effective width of Gaussian distribution of particle fraction over parameter μ
$\varphi(x)$	dimensionless profile of lateral force
$\psi(qa)$	light scattering indicatrix
$\omega = 2\pi f$	cyclic frequency; $\omega_0 = qv_{\|\|} = 2\pi f_0$

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SHORT CONFERENCE REPORT

18th INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY, AMSTERDAM, NETHERLANDS, SEPTEMBER 23–28, 1990

The symposium was attended by about 850 participant who where welcomed by reasonably pleasant and typical Dutch early-autumnal weather. The level of the contributions was excellent and the social events were on the usual Dutch scale — any thought of economic recession was at that time a clogsized cloud on the horizon. Some indication of the jollity is presented here.

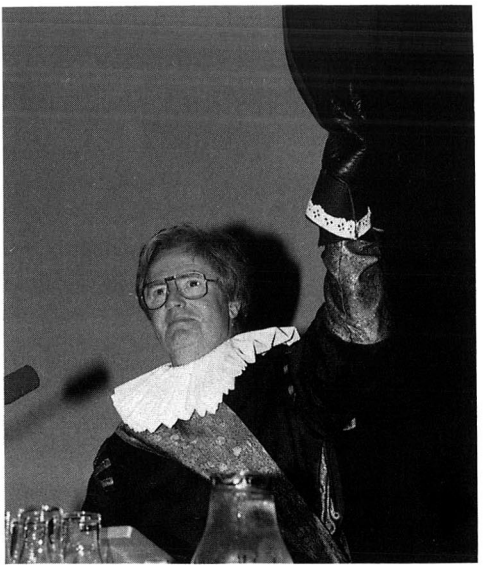

Fig. 1. Udo Brinkman in full seventeenth-century glory waving regally to his admirers after delivering a staccato and extended rapid-fire speech.

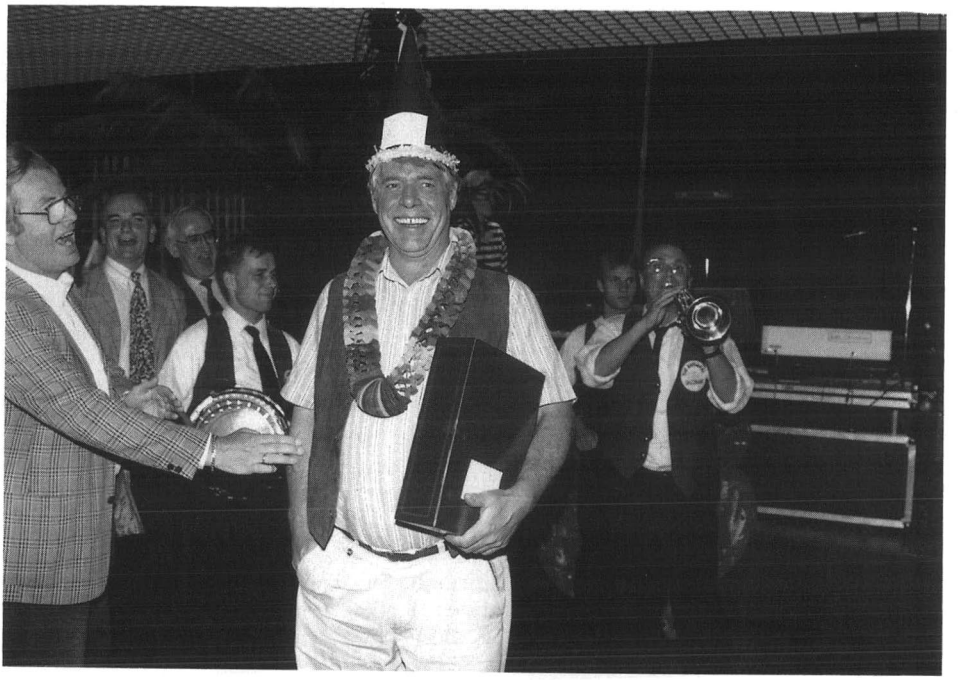

Fig. 2. A very Dutch corner at one of the parties. A less-formal Udo appears to wish to restrain the characteristic ebullience of a Hawaiian Johan Kraak wearing a curious hat for his fiftieth birthday. Carl Cramers and Hans Kragten are either just much amused, or singing, or both.

Fig. 3. A wild dance demonstration by Ira Krull at the same party with the maidens modestly turning their backs to the camera.

Fig. 4. A rare photograph of Guy van Dam (Elsevier's promotion manager) attending a social event. He even appears to be enjoying it, with John Knox and Josef Kříž, in the non-alcoholic section.

Fig. 5. One of the rooms in the West Indisch Huis during the Elsevier evening. Amongst others we see Karin Markides, Fritz Erni, chairman of the 1991 HPLC Symposium in Basel, Barry Karger, Peter Hupe and Ad de Jong.

Fig. 6. Fred Regnier and Hans Laeven smile happily although their companion appears already to have a headache.

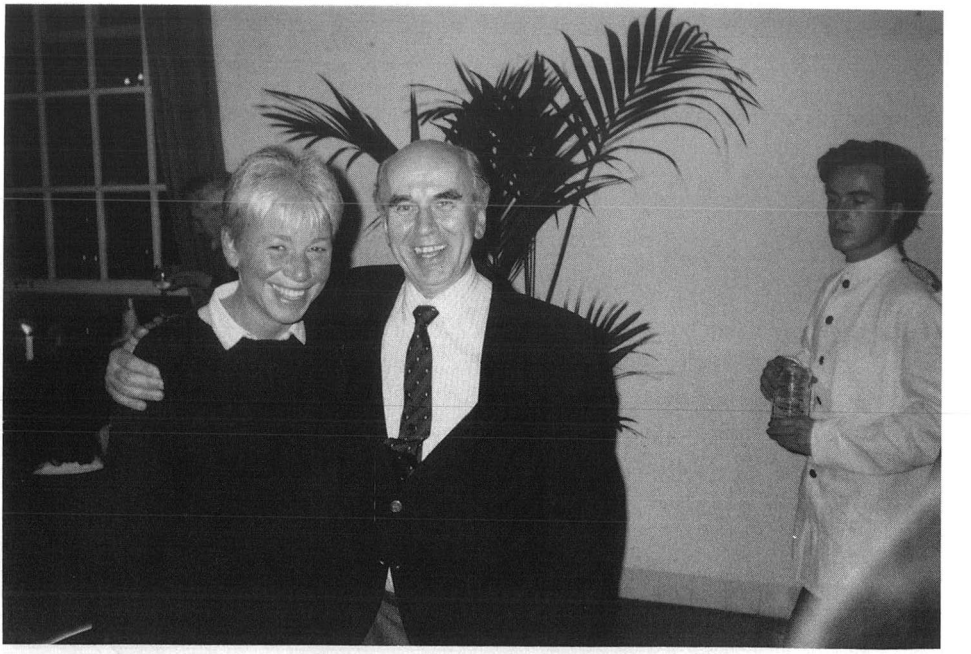

Fig. 7. Trees Roof-Kramer is clasped by Karel Macek. She has assisted in the smooth production of the Biomedical Applications Section of the Journal of Chromatography for very many years.

Fig. 8. The success of the evening is cemented by the intense eye-to-eye contact of Ljudmila Kolomiets and Herman Frank.

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Cumulative Indexes, Vols. 501–550				551/1 + 2				
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